DEVELOPMENTAL PROGRAMMING: PRENATAL ANDROGEN EXCESS DISRUPTS ANTRAL FOLLICLE FUNCTION IN SHEEP

BY

Jonathan Charles Grant, B.Sc (Hons), M.Sc

THESIS SUBMITTED TO THE
UNIVERSITY OF GLASGOW FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

JANUARY 2011

INSTITUTE OF BIODIVERSITY, ANIMAL HEALTH AND COMPARATIVE MEDICINE
UNIVERSITY OF GLASGOW
GARSCUBE CAMPUS
GLASGOW
G61 1QH

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENTS

There are many people, too many to mention in person, who have helped me in various capacities throughout this project. Although I will acknowledge a few key persons, I am so very grateful to all of the academic, technical and administrative staff who aided me at the Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, UK

First and foremost, I must extend a special thank you to my supervisors, Dr Monika Mihm and Dr Jane Robinson for all their abiding help, support and patience, especially throughout the preparation of this thesis. I can state without question that I am extremely fortunate to have you both as my PhD supervisors, and I shall be eternally grateful to you both for the kindness and understanding you have given me over the last 4 years.

My thanks also various members of my research department, formerly known as the Department of Cell Sciences – Dr Paul Baker, Dr Dale Sandercock, Dr Michelle Bellingham, Dr Ana Montiero, Janette Bonnar, Lynne Fleming and Ian Gibson for all their help, technical expertise and good natured humour. Thanks to my office mates Dr Karen Spencer and Dr Tony Robertson for all their banter! A special mention to Dr Peter Hastie for his statistical advice and his ever willing eagerness to help me throughout my research project.

Last, but certainly not least, special thanks must go to all of my family, my beautiful girlfriend Kirsten and to my study partner, Eddie the cat. Mum, Dad and my identical twin brother Paul, without your unconditional love and support I would never have completed this task. I have been blessed to have as supportive parents as my Mum and Dad. Paul thanks for listening to me moan and for all the banter during the hour long drive into the university over the last few years. Kirsten thanks for your patience, especially over the last few months – I promise that I will make it up to you. It is for them that I have completed this thesis.

LIST OF CONTENTS

LIST OF FIGURES Page

LIST OF TABLES Page

17β-HSD	17β-hydroxysteroid dehydrogenase
20α-HSD	20α-hydroxysteroid dehydrogenase
AMH	Anti-Mullerian hormone
ATP	Adenosine 5'-triphosphate
BH	BCL-2 homology
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3',5'-monophosphate
CASP3	Caspase 3
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum / Corpora lutea
CYP11A	Cytochrome P450 side-chain cleavage
CYP17	Cytochrome P450 17α-hydroxylase 17,20 lyase
cpm	Counts per minute
cRNA	Complementary ribonucleic acid
CYP11A	Cytochrome P450 side-chain cleavage
CYP19A1	Cytochrome P450 aromatase
Da	Daltons
DHT	5α-Dihydrotestosterone
DNA	Deoxyribonucleic acid
E2	Oestradiol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Oestrogen receptor(s)
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
Follicular Fluid	FF
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor(s)
GC	Granulosa cells
GDF-9	Growth differentiation factor-9
GH	Growth hormone
GnRH	Gonadotrophin-releasing hormone
HSD3B1	3β-hydroxysteroid dehydrogenase
IGF	Insulin-like growth factor(s)
IGF-R	Insulin-like growth factor receptors
IGF-I	Insulin-like growth factor I
kb	Kilobases
kDa	Kilodaltons
KL	Kit ligand
LH	Luteinising hormone
LHR	Luteinising hormone receptor(s)
mRNA	Messenger ribonucleic acid(s)
NCBI	National Center for Biotechnology Information
NHPP	National Hormone and Peptide Program
nt	Nucleotides
P4	Progesterone
PB	Phosphate buffer
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction

$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
RIA	Radioimmunoassay
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription – polymerase chain reaction
StAR	Steroidogenic acute regulatory protein
TC	Thecal cells
TGF	Transforming growth factor(s)
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TP	Testosterone Propionate
qPCR	Quantitative polymerase chain reaction
ZP	*Zona pellucida*

ABSTRACT

The experiments detailed in this thesis were conducted to investigate the effects of the prenatal environment, specifically excess aromatisable testosterone and the non-aromatisable 5α-dihydrotestosterone, on early adult reproductive function in female sheep, focusing on ovarian antral follicle development.

In Chapter 3, blood samples and the largest antral follicles were obtained from sheep prenatally exposed to excess testosterone propionate (TP) (tissue collection over 5 years) and 5α-dihydrotestosterone (DHT) (tissue collection in 1 year) and controls. The aim was to determine the effect of prenatal androgen treatment on ovarian function in the young adult ewe, specifically on circulating steroid concentrations (oestradiol and progesterone), ovarian weight, and on large antral follicle characteristics (follicle diameter, follicular fluid oestradiol, progesterone and testosterone concentrations). Prenatal androgenisation by TP reduced the proportion of ewes undergoing at least one reproductive cycle during the first breeding season. Compared with controls, ovarian weight and peripheral concentrations of oestradiol were increased in ewes prenatally treated with TP. In addition, the largest (generally two) antral follicles recovered from ovaries of ewes exposed prenatally to aromatisable testosterone demonstrated an increase in size, and an increase in follicular fluid oestradiol and progesterone, both markers of follicle differentiation.

In Chapter 4, ovaries were harvested from control and TP androgenised ewes at 10- and 22-months of age to investigate the effect of prenatal treatment with TP on small antral follicle health and steroidogenesis in adulthood. In early antral follicles following antrum formation follicle health was improved in 10 month old ewes and more follicles had acquired the ability to produce oestradiol in 22 month old prenatally TP treated ewes.

In Chapter 5, the (generally two) largest antral follicles were obtained from ovaries from control and prenatally androgenised (TP) 10 and 12 month old ewes to determine the mRNA expression profile for differentiation, steroidogenic, survival and apoptotic genes in granulosa cells. Granulosa cells of TP ewes demonstrated increased mRNA expression for *LHR* and *HSD3B1* and reduced mRNA expression for *FSHR*. Gene expression levels of various proliferation and apoptotic makers in granulosa cells were similar between control and androgenised ewes.

In Chapter 6, granulosa cells were isolated from the (generally two) largest antral follicles recovered from control and prenatally androgenised (TP and DHT) ewes of 10-months of age. These were cultured *in vitro* under different gonadotrophin conditions to determine if granulosa cell steroid production is intrinsically different between androgenised and control ewes, or whether the external (hormonal) environment causes the differences detected in antral follicle function. Unfortunately, investigation into the mRNA expression profiles of FSH- and LH- responsive genes in granulosa cells after culture was not possible due to low cell numbers. Oestradiol production was increased independent of gonadotrophins, and progesterone production in response to LH was increased in cells from TP-treated ewes compared with control cells, while prenatal androgenisation by DHT resulted in reduced granulosa cell oestradiol production compared with cells from both control and TP androgenised ewes. Prenatal androgenisation through DHT resulted in reduced granulosa cell progesterone production when compared with cells from TP treated animals.

The results presented in this thesis provide evidence that prenatal androgen treatment causes programming of adult ovarian function in sheep. Exposure to excess TP results in altered large antral follicle function such as increased growth and steroidogenesis. Specifically, androgenisation using TP and not DHT leads to increased steroidogenesis

(oestradiol and progesterone) in large antral follicles, therefore these abnormalities are programmed through oestrogen action. Increased *LHR* and reduced *FSHR* mRNA expression, together with an earlier acquisition of steroidogenic capability provide evidence for the first time that follicles recovered from TP treated ewes are further differentiated. Future studies should be directed towards establishing whether intra-ovarian levels of gene expression equate with protein production. Furthermore, further studies need to address any molecular changes in somatic cells of early antral follicles. Finally, a study abolishing gonadotrophins using a GnRH antagonist using supplementation treatments should be performed, to truly determine if it is indeed LH that is responsible for abnormal antral follicle growth and development in prenatally TP-treated ewes.

CHAPTER 1. REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Successful reproduction is, obviously, key to the survival of any species and environmental perturbations which impact on this process will have wide ranging effects on the health and wellbeing of a population. While the environment may influence an organism's reproductive physiology at any stage of its life cycle, early developmental processes, such as those occurring during *in utero* life, are particularly susceptible. For many years it has been recognised that gestational exposure to steroids programmes normal sexually dimorphic physical development (Jost *et al.*, 1973). However, exposure to abnormal concentrations of steroids (for example as a consequence of steroid-releasing tumours or congenital adrenal hyperplasia) can lead to physiological dysfunction in later life. This subject has recently received global attention, as we are constantly exposed to chemicals in our environment that can bind to and activate steroid receptors, the so called 'steroid mimetics' or 'endocrine disrupters' and it has been proposed that abnormal exposure of the foetus to these chemicals, or to abnormal endogenous steroid concentrations, can programme disease states in later life leading to sub or infertility.

Polycystic ovarian syndrome (PCOS) is the most common female endocrine disorder of women of reproductive age affecting 5-10% of women between the ages of 12 and 45 (Franks, 1995; Franks *et al.*, 2008). Despite extensive research the aetiology of PCOS is poorly understood. However, it is believed that a subset of women with this condition have been exposed to elevated concentrations of androgen *in utero* (Franks *et al.*, 2006). This hypothesis, and a quest to understand the consequences for the reproductive axis of exposure of the female foetus to androgens, has led to the development of several animal models, including the *in utero* androgenised ewe, and this model will be described in detail in section 1.5. During the last few decades, considerable advances have been made with respect to the control mechanisms that regulate female reproduction. However, the precise mechanisms, particularly those of intra-ovarian origin, that control follicular growth and development remain elusive. An understanding of the basic mechanisms that control follicular development will help us to address common causes of infertility. Therefore, a major challenge for research scientists is to determine the precise mechanisms that underpin follicular growth and development.

The overall aim of the studies presented in this thesis is to further characterise the effect that the prenatal environment, specifically excess androgens has on reproductive function, and determine the ovarian features that are programmed before birth. Results gained from

using the prenatally androgenised sheep model will contribute to a greater understanding of the mechanisms that control ovarian follicle growth and development. Specifically, the studies aim to investigate whether androgenisation *in utero* causes alterations in antral follicle function, hormone responsiveness and the expression of specific differentiation, steroidogenic, survival and apoptotic factors.

1.2 SHEEP AS A MODEL FOR INVESTIGATING FOLLICLE GROWTH AND DEVELOPMENT

It is important to address our choice of an ovine model for studying the programming of ovarian follicle growth and development, rather than using the human or more popular rodent models. Availability of suitable ovarian tissue is a major restriction in the study of follicle development in humans, therefore single- or double-ovulatory species like the sheep represent a physiologically relevant model to elucidate the basic reproductive mechanisms before embarking on more focused clinical investigations (Campbell *et al.*, 2002). In smaller mammals (mouse, rat,) multiple follicles mature into preovulatory follicles (polyovulatory), in contrast to some breeds of sheep where, like humans, only one follicle matures into the dominant/preovulatory follicle. Further, sheep have a reproductive cycle that more closely resembles that of women, with distinct luteal and follicular phases which is not the case in the rodent, in which the cycle is much shorter in duration (Marcondes *et al.*, 2002). The neuroendocrine control of the ovine oestrous cycle has been intensively studied, therefore, there is a wealth of background information on which to base future studies. An additional attraction in using the sheep as a model is that they, like humans, are long-lived and have a relatively long gestation period (147 days), during which time ovine and human foetuses show a similar timing and sequence of ovarian development (Juengel *et al.*, 2002; Sawyer *et al.*, 2002). Specifically, in humans and sheep, formation of the follicle pool is completed prenatally (which dictates the longevity of adult female fertility) (Baker, 1963), thus mechanisms in primordial follicle formation elucidated in the sheep can be applied to the human (Campbell *et al.*, 2002). In contrast, in the rat and mouse follicular formation does not begin until the perinatal period and is completed within one to two weeks of birth. A final attraction of the ovine model is that the duration of follicle growth and development (primary follicle to ovulation) is similar (5-6 months) in sheep and humans (Campbell *et al.*, 2002). All of these features make using the sheep a highly appropriate choice as a model for studying ovarian follicle growth and development.

1.3 Endocrine Control of the Oestrous Cycle in Sheep

The sheep is a polyoestrous seasonal breeding mammal with a reproductive cycle usually lasting 16-18 days that results from the co-ordinated interaction of three main reproductive tissues, the brain, pituitary gland and the ovary (Goodman, 1994). Reproductive function is controlled by a variety of physiological and hormonal factors, which in turn are responsive to a range of environmental factors including other animals, light, temperature and nutrition. Sheep are seasonal breeders with distinct periods of cyclicity observed during the autumn and winter months, thus ensuring that lambs are born at a time when climatic conditions are favourable and when adequate levels of nutrition are available. Periods of cyclicity (in the absence of pregnancy) are interspersed with periods of acyclicity (anoestrus) during the spring and summer months. While reproductive cycles usually last 16-18 days there may be variation, particularly at the beginning and end of the breeding season (Hafez, 1952).

The ovine oestrous cycle can be divided into two functionally distinct parts; a relatively short follicular phase and a longer luteal phase. The follicular phase typically lasts for 2-3 days and is the period where the selected preovulatory follicle(s) secretes high concentrations of oestradiol (Baird, 1978) and enters the final stages of maturation, ultimately culminating in ovulation approximately 30-40h after the onset of behavioural oestrus (Hutchinson *et al.*, 1987). The luteal phase typically lasts for 14-15 days and is hormonally dominated by progesterone secretion from the *corpus luteum* (CL) that has developed from the ovulatory follicle. In the absence of pregnancy, luteolysis is initiated 14-15 days after ovulation, signalling the end of the luteal phase and the commencement of the next follicular phase. In the absence of a conceptus, it is essential that any CL regress in order to maintain cyclicity. This is achieved by the direct actions of a luteolytic signal. Conversely, in the pregnant animal the luteolytic signal is absent, and progesterone secretions from the CL remain high, which presents a negative feedback at the hypothalamo-adenohypophyseal level, and subsequently terminal follicle growth and ovulation is inhibited. The primary luteolytic signal in ewes is $PGF_{2\alpha}$ (Goding, 1974). Luteolysis generally occurs between days 12-15 of the oestrous cycle (Bazer *et al.*, 1997) and it is characterised by (i) a marked decrease in progesterone secretion; followed by (ii) a marked decrease in luteal size and weight.

1.3.1 The Hypothalamo-Pituitary Ovarian Axis

The hypothalamo-pituitary-ovarian axis is responsible for controlling reproduction and oestrus in sheep, and the disruption or removal of any one component alters the entire reproductive activity of the female. This axis is a tightly interrelated system whereby

normal functioning of the ovary (follicle development, ovulation) is driven almost entirely by the pituitary hormones luteinising hormone (LH) and follicle stimulating hormone (FSH), whose secretion in turn is controlled via the hypothalamic hormone gonadotrophin releasing hormone (GnRH), and the feedback of steroid hormones.

1.3.1.1 Hypothalamic control

The hypothalamus is located at the base of the brain and it is connected to the pituitary gland by the hypophyseal stalk, which contains numerous nerve terminals and capillaries. The hypothalamus, via the hormones it secretes, has several effects on key physiological processes such as temperature homeostasis, appetite and heart rate. However, of primary interest in this thesis is the key role the hypothalamus plays in the regulation of reproduction. Gonadotrophin-releasing hormone (GnRH) is the major hormone released by the hypothalamus that regulates reproduction. It has also been found to be involved in the control of ovine oestrous behaviour (Caraty *et al.*, 2002). In the sheep there are about 2000 neurones that synthesise and release GnRH and they are located in a loose continuum largely in the medial preoptic area sending their axons to the median eminence (Lehman *et al.*, 1986; Caldani *et al.*, 1988). This distribution is similar to that observed in other species such as the rodent and rhesus monkey (Silverman *et al.*, 1994). The scattered distribution probably reflects the fact that GnRH neurones do not originate in the brain but migrate from the olfactory placode during development (Schwanzel-Fukuda & Pfaff, 1989). In the sheep this migration is complete by about day 52 of gestation and immunoreactive fibres are located in the median eminence by day 66, increasing in density with time (Caldani *et al.*, 1995). Thus, it is probable that the anterior pituitary gland is under the regulation of hypothalamic factors from the mid point of gestation. GnRH is secreted in a distinctive pulsatile manner (Clarke & Cummins, 1982), which is a reflection of the synchronised activity of the GnRH neurons in the hypothalamus (Catt, 1999). In more recent years the presence of a hypothalamic factor that inhibits gonadotrophin release from the pituitary gland has been identified. The discovery of gonadotrophin inhibitory hormone (GnIH), was made in the Japanese quail (Tsutsui *et al.*, 2000) and the mammalian orthologue RFamide-related peptide 3 (RFRP-3) has recently been described in the ovine hypothalamus (Qi *et al.*, 2009). The precise role that inhibitory peptides might play in the control of LH and FSH secretion is currently unknown.

1.3.1.1.1 Patterns of GnRH secretion

Attempts to monitor the activity of the GnRH neurones have been made since the 1970's when increased electrical activity in the hypothalami of monkeys was found to be associated with changes in episodic LH release (Wildt *et al.*, 1981). Since this time more

direct measurements of the release of GnRH have been made via push pull perfusion (Levine & Ramirez, 1982) or microdialysis (Sisk *et al.*, 2001) in the median eminence or direct measurement of the decapeptide in the cerebrospinal fluid (CSF) or the portal blood of several species (rat: (Sarkar *et al.*, 1976), sheep: (Clarke & Cummins, 1982; Skinner *et al.*, 1995), Rhesus monkey, (Pau *et al.*, 1993), cow: (Yoshioka *et al.*, 2001), horse (Irvine & Alexander, 1987)). It has become clear that each pulse of LH is preceded by a pulse of GnRH although each GnRH episode does not necessarily result in LH secretion. The pattern of GnRH secretion has also been shown to vary throughout the course of the oestrous cycle and during the yearly cycle of seasonally breeding species. Specifically, throughout the luteal phase of the ovine oestrous cycle, GnRH secretion is characterised by high amplitude, low frequency pulses (Clarke *et al.*, 1987; Moenter *et al.*, 1991). However, during the transition to the follicular phase, and as a result of a decrease in plasma progesterone concentrations, GnRH pulse frequency increases (Clarke *et al.*, 1987; Moenter *et al.*, 1991). As plasma oestradiol concentrations progressively increase throughout the follicular phase (due to the increase in production of oestradiol from the dominant follicle or several preovulatory follicles), this results in a continued increase in the GnRH pulse frequency together with a decrease in pulse amplitude, giving rise to a preovulatory surge in GnRH secretion (Moenter *et al.*, 1993). During the transition into anoestrus the frequency of GnRH pulses decreases markedly as a result of the change in melatonin secretion, and they remain low for the duration of anoestrus (Barrell *et al.*, 1992), having a dramatic effect on gonadotrophin secretion.

1.3.1.2 The control of the anterior pituitary gland

The pituitary gland is divided into two main subdivisions, the anterior and posterior pituitary gland which have different embryological origins first documented by Martin Heinrich Rathke in 1838. Specifically the anterior pituitary arises from an outgrowth of the oral cavity (Rathke's pouch) while the posterior pituitary is formed from a diverticulum from the floor of the brain and is, therefore, neural tissue. GnRH is transported to the anterior pituitary gland, where it has its action, in the hypothalamo-hypophyseal portal system, which is a distinct vascular connection. The main hormones released by the anterior pituitary gland that regulate reproduction are the gonadotrophins, namely follicle-stimulating hormone (FSH) and luteinising hormone (LH), primarily influencing ovarian function and steroid hormone secretion. FSH is the major key regulatory hormone involved in follicle growth and development, and LH concomitant with FSH (to a much lesser extent) are the key hormones involved in terminal preovulatory follicle maturation and ovulation. FSH plays a major role in growth and maturation of ovarian antral follicles, granulosa cell proliferation, prevention of atresia, induction of aromatase (the enzyme

responsible for oestradiol production), and induction of LH and FSH receptors in the ovary (Richards, 1994).

The pituitary gland is very active during development and this activity reaches a peak during mid gestation in the sheep followed by a period of relative inactivity during the pre-pubertal period (Brooks *et al.*, 1995). Interestingly there is a sex difference in prenatal pituitary activity such that gonadotrophin release is lower in male foetuses in mid gestation due to the negative feedback actions of testosterone (Sklar *et al.*, 1981; Matwijiw & Faiman, 1989). Using immunocytochemistry, the common alpha subunit of the gonadotrophins and the beta subunit of LH were detected in a small portion of ovine pituitary cells at day 70 of gestation (earliest stage investigated) (Thomas *et al.*, 1993). However, the beta subunit of FSH was not detected until some 30 days later. Thus, it appears that the hypothalamus is competent to release GnRH from the middle of gestation and gonadotrophs are capable of synthesising the gonadotrophins from an early stage of foetal life. GnRH receptors must also be present because GnRH has been shown to be involved in the development of the pituitary gland. Specifically, the implantation of a GnRH agonist (buserelin) subcutaneously into 70 day old foetuses markedly inhibited LH and FSH release at day 110, reduced the expression of mRNA for the beta subunits of LH and FSH and substantially lowered testicular weight (but not ovarian weight) at birth (Brooks and Thomas, 1995). In addition the reproductive axis appears to be responsive to some elements of steroid feedback by mid gestation suggesting that perturbations of the reproductive axis by either endogenous or exogenous factors will have consequences for all levels of the reproductive axis.

1.3.2.1 Patterns of Gonadotrophin Secretion

The patterns of the synthesis and release of LH and FSH diverge in several physiological situations. LH is secreted into the peripheral circulation, and like GnRH, it is secreted in a pulsatile manner. It is generally accepted that LH surge generation is dependent on a sustained increased secretion of GnRH into the pituitary portal blood system (Clarke *et al.*, 1987; Moenter *et al.*, 1991). Additionally, evidence in sheep indicates that oestradiol plays a key role in sensitising the pituitary gland to GnRH, and this is also an integral component of LH surge induction (Clarke & Cummins, 1984; Kaynard *et al.*, 1988). LH, and to a much lesser extent FSH, are the hormones that play the major role in terminal follicle/oocyte maturation (oocyte nuclear maturation to metaphase I and ovulation (Hafez, 1999)). This is achieved via a massive preovulatory LH surge associated with a sustained surge of GnRH (Goodman, 1993). This preovulatory LH surge consists of a combination

of high frequency, low amplitude pulses of LH secretion (Rawlings & Cook, 1993), where the development and progression of the LH surge in the ewe depends entirely upon GnRH stimulation throughout its time course (Clarke *et al.*, 1987; Moenter *et al.*, 1991; Evans *et al.*, 1996).

LH secretion throughout the oestrous cycle (Figure 1.1)

Stage of the oestrous cycle has a dramatic effect on LH secretion. During the luteal phase, similar to GnRH secretion, LH secretion is tonic, being relatively low and infrequent. In this phase of the oestrous cycle LH pulses follow GnRH pulses (Clarke *et al.*, 1987). Tonic release of LH is generally observed as basal peripheral levels of 0.1-2.0ng/ml, interspersed with small episodes (5-15ng/ml) that persist for approximately 30 minutes, and occur every 3-12 hours (Baird *et al.*, 1976; Hauger *et al.*, 1977). Following luteolysis, and hence during the follicular phase, tonic secretion of LH continues. However, as the follicular phase progresses, the pulse frequency increases dramatically where the time between pulses can decrease to as little as 40 minutes and this typically lasts for 10-12 hours. Similar to the pattern of GnRH secretion during the follicular phase, LH pulse amplitude also decreases, yet overall, levels of LH secretion continue to increase with each pulse of LH preceded by a pulse of GnRH (Clarke & Cummins, 1982). This high frequency, low amplitude sustained period of LH secretion culminates in the preovulatory LH surge.

FSH secretion throughout the oestrous cycle (Figure 1.1)

Similar to LH, FSH varies depending on stage of the oestrous cycle. Throughout the oestrous cycle of the ewe, generally, there are four peaks of bioactive FSH (Phillips *et al.*, 1994). Two of these peaks occur during the luteal phase, and are between 4-6 days apart (Bister & Paquay, 1983; Campbell *et al.*, 1991b), with the second peak, in particular, stimulating the development of large oestrogenic follicles (Souza *et al.*, 1997). A third peak of FSH occurs, coincident with the preovulatory LH peak, denoting the preovulatory FSH surge (Phillips *et al.*, 1994). Finally, a fourth FSH peak occurs shortly following the preovulatory FSH surge, and it is of slightly greater duration, but is of lower amplitude (Phillips *et al.*, 1994).

1.3.1.3 Ovarian Control

The third element in the reproductive axis, the ovary, produces three main groups of steroid hormones – progestins, oestrogens and androgens. The primary gonadal steroids in the ewe, progesterone, oestradiol-17β, testosterone and androstenedione are synthesised from cholesterol (Withers, 1992). Large antral ovarian follicles secrete oestradiol, whereas

in the ovary the major source of progesterone is the *corpus luteum* (CL), a temporary endocrine structure that is formed following the release of the oocyte from the follicle during ovulation.

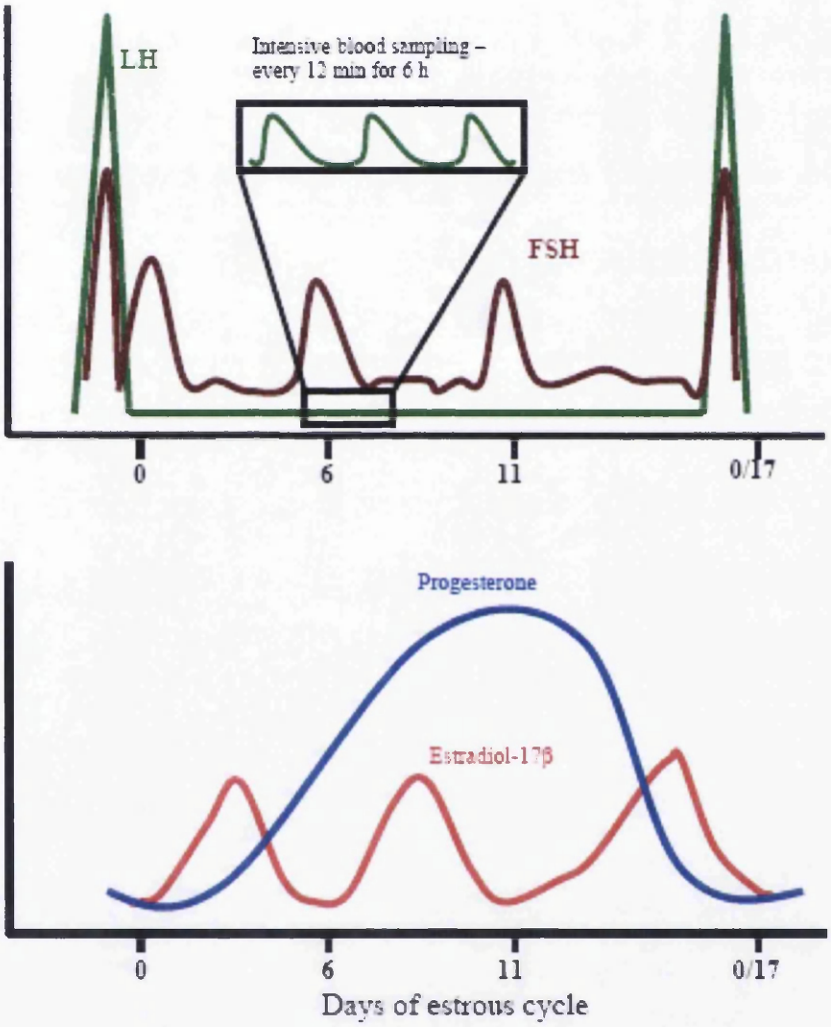


Figure 1.1 Schematic representation of serum profiles of LH and FSH (top panel), and estradiol-17β (oestradiol) and progesterone (bottom panel) throughout an oestrous cycle in the ewe (x-axis: d0 = day of ovulation, y-axis: relative concentrations of hormones). Except for high concentrations during the preovulatory surge, serum LH concentrations remain basal throughout the luteal phase of the cycle. Pulses of LH secretion are detectable in frequently collected blood samples. FSH secretion remains almost non-pulsatile and periodic peaks in FSH secretion occur once every 4-5 d throughout the oestrous cycle. Periodic peaks in oestradiol secretion also occur, but they tend to coincide with nadirs in serum FSH concentrations. Serum progesterone concentrations increase from day 0 to day 11 and then reach a nadir by day 15 after ovulation. Figure adapted from Duggavathi (2004) and based on data from (Pant et al., 1977; Bartlewski et al., 1999; Evans, 2003).

Oestradiol and Progesterone (Figure 1.1)

Patterns of oestradiol secretion have been difficult to ascertain over the years due to the low concentrations of this hormone in peripheral circulation of the sheep. Typical peripheral concentrations are generally between 1-10pg/ml (Deaver & Dailey, 1983). As the follicular phase progresses over a period of 2-3 days oestradiol levels rise. This arises due to a decrease in progesterone concentrations following luteolysis and the coinciding rise in pulsatile LH secretion (Baird *et al.*, 1976). It is generally the largest non-atretic preovulatory follicle(s) that is the source of the majority of oestradiol secretion (Hay & Moor, 1975). Oestradiol levels then reach a peak at the time of the onset of the preovulatory LH surge, coincident with the onset of behavioural oestrus, where they then begin to decline and return to basal levels.

In the ovary, the CL is responsible for the majority of progesterone secretion and synthesis. Progesterone concentrations vary throughout the oestrous cycle. For the first 3 days following ovulation serum progesterone levels are low. However, these then gradually increase reaching a maximum concentration of between 1.5-3ng/ml by day 8-9 depending on the breed of sheep (Bindon *et al.*, 1979) and stage of the breeding season. This progesterone concentration will be maintained throughout the luteal phase of the cycle until about day 14 when, in the absence of pregnancy, it will fall dramatically to basal levels within 24-48 hours. This fall in progesterone is as a result of the active destruction of the CL by prostaglandin $PGF_{2\alpha}$ which is produced from the uterus (McCracken *et al.*, 1970). In the pregnant ewe, high progesterone concentrations from the CL will be maintained until approximately mid-gestation [between days 60-80], from when on placental progesterone production increases dramatically, increasing peripheral concentrations of progesterone even further until the point of parturition (Denamur, 1968).

Inhibin, Activin and Follistatin

It has become apparent over the years that, in addition to steroids, peptides derived from the ovary, such as inhibin, activin and follistatin have key regulatory roles in controlling reproduction. Inhibin and activin are structurally-related peptides, which are functionally diverse, whereas follistatin is an activin-binding protein that is functionally (but not structurally) similar to inhibin (Findlay, 1993). Inhibins and activins are members of the TGF-β superfamily. There are two forms of inhibin, inhibin A (α-βA) and inhibin B (α-βB), which are generated as a result of the fact that inhibins are dimers of a unique α subunit linked to either a βA or βB subunit. Furthermore, three forms of activin are generated following dimerisation of β subunits, and they are termed activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB) (Webb *et al.*, 1994; Knight & Glister, 2001).

Follistatin is encoded by a single gene, although there are numerous isoforms as a result of alternative mRNA splicing and post-translational modifications (Shimasaki *et al.*, 1988; Sugino *et al.*, 1993)

Inhibin is found in high concentrations in follicular fluid and appears to act in synergy with oestradiol to inhibit tonic FSH secretion. It is thought that oestradiol acutely inhibits FSH secretion in the short-term, whereas inhibin, may control FSH secretion in the medium to longer term, by setting the overall level of negative feedback (Baird *et al.*, 1991; Campbell *et al.*, 1991b).

Inhibin concentrations tend not to fluctuate enormously throughout the oestrous cycle although they are found to increase and be highest during the late follicular phase (Campbell *et al.*, 1990; Findlay *et al.*, 2000; Knight *et al.*, 1998). This is presumably as a result of production from large preovulatory oestrogenic follicles, although secretion has also been demonstrated from non-oestrogenic large follicles and small follicles, albeit at lower concentrations (Campbell et al., 1991). It is only the follicle, and not the CL, that seems to be the major source of inhibin production (Campbell et al., 1991) in the sheep ovary.

The situation regarding the regulatory roles of activin and follistatin in controlling reproduction is less clear. Activin is secreted by granulosa cells within the ovary (Drummond *et al.*, 1996; Findlay *et al.*, 2001) but also acts as an autocrine and/or paracrine factor within the pituitary gland to promote FSH production and release (Ling *et al.*, 1988; Carroll *et al.*, 1989). Follistatin was initially isolated from follicular fluid and identified as a protein that inhibited FSH secretion. Since then its primary function has been determined to be the binding and bioneutralization of activin (Knight, 1996), thus indirectly controlling FSH secretion.

Initially, it was suggested that follistatin and activin were unlikely to act in an endocrine manner given that peripheral concentrations of follistatin throughout the cycle are relatively constant (Khoury *et al.*, 1995) and there is very little free activin present in the circulation (McConnell *et al.*, 1998). However, studies in sheep have determined that the suppressive effects of follistatin are mediated, in part, by neutralising circulating activin (Padmanabhan *et al.*, 2002).

1.4 Follicle Growth and Development

Follicle growth and development describes the growth of an ovarian follicle as it progresses through the different stages of development from the time that it is recruited from the primordial pool, formed during oogenesis, until the time that it either ovulates or, in the case of the vast majority of follicles, becomes atretic. In domestic ruminants, as in many other mammalian species, follicle growth and development are controlled by a complex system, which incorporates both endocrine mechanisms involving the HPO axis, as well as other intra- and extra-ovarian factors. The whole process can best be described using the terms proposed by Hodgen (1982) – Recruitment, Selection and Dominance.

1.4.1 Ontogeny of Follicle Growth and Development in Sheep

1.4.1.1 Oogenesis

Oogenesis is defined as the formation, development and maturation of female gametes (Crisp, 1992). In sheep, oogenesis commences early in foetal life (day 23-24 of gestation) with the arrival of 1,000-2,000 stem cells termed primordial germ cells (PGC) (Crisp, 1992). These primordial germ cells originated in the primitive ectoderm and migrated during early development into the genital ridges. By approximately day 40 of gestation, all of the germ cells present in the ovary are surrounded by somatic cells and enclosed into sex cords that are located in the cortex (Van den Hurk *et al.*, 1995, 1997); they are now mitotically-active oogonia (Smith *et al.*, 1993, 1997). These germ cells, which have differentiated into oogonia, proliferate via a series of mitotic cycles until approximately day 90 of gestation, where it is thought that they may number 600,000 (Crisp, 1992). At this time, germ cells in foetal ovaries are predominantly oogonia or isolated oocytes (70-90%) with few primordial and primary follicles present (Clark *et al.*, 1996; Smith *et al.*, 1993, 1997). Germ cells are only transformed into oocytes when mitotic proliferation is completed, which usually begins by day 75 and is generally completed by day 100 of gestation in sheep (Smith *et al.*, 1993). However, meiosis at this stage is not complete and the oocyte becomes arrested in the diplotene stage of the first meiotic division (Bacharova, 1985; Hirshfield, 1991; Van den Hurk et al., 1997; Webb et al., 1999).

1.4.1.2 Quiescent primordial follicle population

The formation of a large reserve of quiescent primordial follicles, residing in the outer cortex of the ovary, is classified as the first stage of follicle development (Hirshfield, 1991). The stimulus for the transformation from oogonia to oocytes is still relatively unknown (Crisp, 1992). 'Naked' oogonia - which lack follicular cell investment, become isolated and surrounded by a layer of flattened follicular cells. This signifies the

appearance of primordial follicles (Crisp, 1992). Primary follicles, which contain one complete layer of cuboidal granulosa cells, can be observed around day 100 in sheep and by day 120 approximately 19% of the germ cell population may be present in follicles with up to three concentric layers of granulosa cells (Smith *et al.*, 1997).

Primordial follicles contain a single layer of pre-granulosa cells, resting on a basal lamina, which surround an oocyte arrested in the diplotene stage of meiosis. These pre-granulosa cells normally cease to divide and enter a generally prolonged period of quiescence (Hirshfield, 1991). In most mammalian species the number of primordial follicles in the ovaries appears to be fixed by late foetal life or shortly thereafter in early postnatal life (McNatty *et al.*, 1992), and this resultant store of follicles is referred to as the primordial pool. It is from this non-growing pool that follicles are gradually recruited during the reproductive life of the animal. In addition, together with meiotic arrest, large numbers of germ cells are lost through a process of attrition between days 75-90 of foetal life; a process that continues throughout early postnatal life (McNatty *et al.*, 1992).

In sheep, the first primordial follicles appear on about day 75 of foetal life, and by day 135, just prior to birth, approximately 90% of germ cells are in primordial follicles and 4% in growing follicles that may develop to between 0.25 and 0.80mm in diameter, while the remainder are present as isolated oocytes (McNatty *et al.*, 1992; Smith *et al.*, 1997; Sawyer *et al.*, 2002). Pre-granulosa cells encapsulate the majority of oocytes (McNatty *et al.*, 1992). The size of the follicle pool is inherently variable, even between genetically identical animals of the same age (Gosden & Telfer, 1987) and it has been estimated that the number of primordial follicles in young ewes is ca. 0.4-3 x 10^5, with a large between-animal and between-breed variation (Cahill *et al.*, 1979). External factors, such as nutrition can also have an impact and there are data which clearly show that under-nutrition in foetal and/or neonatal life will dramatically reduce the concentration of oogonia and primordial follicles in the ovine ovary (Borwick *et al.*, 1994, 1995; Robinson, 1996; Rae *et al.*, 2001).

1.4.1.3 Morphology of follicle growth

The time it takes for a primordial follicle to progress to ovulatory size is about 180 days and is longer in sheep and cattle compared to most laboratory species. Following a prolonged period of quiescence, the first signs that follicle growth has resumed is a change in granulosa cell morphology from a flattened to a cuboidal appearance, followed by proliferation of the granulosa cells and an increase in the size of the oocyte (Hirshfield, 1991; Paton & Collins, 1992; Braw-Tal, 2002). This is followed by a period of committed

growth and development whereby follicles cannot go back into a quiescent state (Scaramuzzi *et al.*, 1993). Recommencement of growth from the primordial pool is likely to be under the control of intra-ovarian/oocyte-derived factors, and studies in sheep (Cahill & Mauleon, 1980) and cows (Scaramuzzi *et al.*, 1980) have indicated that every day, about 3 and 6 follicles, respectively, grow from the pool of primordial follicles. As they acquire a cuboidal layer of granulosa cells, they become intermediary and then primary follicles. However, it is likely that the exact number of follicles entering the growth phase is dependent on the pool of non-growing follicles (Krarup *et al.*, 1969). As the follicle continues to grow, it attains several distinctive morphological features, namely: a thick acellular *zona pellucida* (ZP) that surrounds the oocyte, the *theca interna* (steroidogenic cells), the *theca externa* (connective tissue cells), a capillary network, a basement membrane and a fluid-filled cavity called an antrum (Hirshfield, 1991).

Creation of an antrum

Follicles do not possess a major pool of extra-cellular fluid from the beginning of their development (Gosden *et al.*, 1988). However, in the latter stages of follicle growth, when the follicle is between 0.2 and 0.4mm in diameter in sheep, fluid-filled spaces develop between the granulosa cells that eventually combine into a single, large, fluid-filled antral cavity (Hirshfield, 1991; Webb et al., 1999). This fluid within this antral cavity is called follicular fluid and is essentially a filtrate of thecal blood (Crisp, 1992). Follicular fluid contains substances from local secretion and metabolism (Gosden *et al.*, 1988); especially steroids, glycosaminoglycans and many other metabolites (McNatty, 1978; Hafez, 1993a). Follicular fluid plays a distinct role in the physiologic, biochemical and metabolic aspects of nuclear and cytoplasmic maturation of the oocyte and the release of the egg from the ruptured follicle (Hafez, 1993a).

1.4.1.4 Cellular proliferation & differentiation

Granulosa cell proliferation increases with increasing follicle size, with maximum follicle growth during the antrum formation stage (Hirshfield, 1991). It is difficult to ascertain the exact rate of proliferation, and subsequent growth of a follicle from one size to another, since the rate of granulosa cell proliferation is affected by a variety of factors. However, as mentioned previously, the full course of follicle growth in adult ruminants is in the region of 180 days (Campbell *et al.*, 1995), compared to rodents, where it is only several weeks. While cellular proliferation is at a maximum during antrum formation, and shortly thereafter, this does decrease dramatically in the preovulatory follicle, where growth is very slow (Hirshfield, 1986). The somatic cells in the mature ovarian follicle display a high

degree of differentiation and a variety of tissue-specific, functional features (Hirshfield, 1991). However, this is not the case in immature follicles early on, which are less differentiated, but highly proliferative.

1.4.1.5 Follicular steroidogenesis

As well as playing a key role in the generation of the preovulatory GnRH/LH surge, oestradiol also plays an imperative intra-ovarian role in preventing atresia (Reilly *et al.*, 1996) and regulating follicle development (Drummond & Findlay, 1999; Schams & Berisha, 2002; Beg et al., 2003). In order that steroids, including oestradiol, can be synthesised by steroidogenic cells, they must first acquire cholesterol either via *de novo* synthesis or by the uptake of cholesterol (Strauss *et al.*, 1981; Gwynne & Strauss, 1982; Brown & Goldstein, 1997). The actual biosynthesis of oestradiol incorporates both the theca and granulosa cell layers. These 2 layers must integrate fully to facilitate the conversion of cholesterol to oestradiol. The conversion of the various precursors depend entirely upon many enzymes, in particular several members of the large cytochrome P450 family of heme-containing enzymes, and hydroxysteroid dehydrogenases (HSD) (Strauss & Penning, 1999) (Figure 1.2). The proposed nomenclature for cytochrome P450 enzymes states that each family member is designated CYP followed by a unique number identifier that is a function of the protein's catalytic activity (e.g. CYP11A for cytochrome P450 side-chain cleavage enzyme) (Strauss & Penning, 1999).

The rate-limiting step in gonadal steroidogenesis is the transfer of cholesterol from the relatively cholesterol-rich outer mitochondrial membrane to the cholesterol-poor inner mitochondrial membrane (Stocco & Clark, 1996; Stocco, 2000). This regulated step in steroid production is catalyzed by the steroidogenic acute regulatory protein (StAR) (Lin *et al.*, 1995; Arakane *et al.*, 1998). Following transportation to the inner mitochondrial membrane, cholesterol is converted to pregnenolone, and CYP11A catalyses this conversion (Strauss & Penning, 1999). CYP11A is localised to both granulosa and theca layers in the sheep follicle (Huet *et al.*, 1997). However, the next 2 steps take place almost exclusively in thecal cells. Firstly, CYP17 catalyses the conversion of pregnenolone and progesterone to dehydroepiandrosterone and androstenedione. Secondly, 3β-HSD (HSD3B1) catalyses the conversion of pregnenolone into progesterone, 17α-hydroxypregnenolone into 17α-hydroxyprogesterone, and dehydroepiandrosterone into androstenedione (Conley *et al.*, 1995; Huet *et al.*, 1997; Strauss *et al.*, 1999). Type I 17β-HSD catalyses the conversion of the weak androgen, androstenedione, to the more potent androgen, testosterone (Strauss & Penning, 1999). Finally, testosterone and/or

androstenedione are aromatised into oestradiol in the granulosa cells by CYP19 (Huet *et al.*, 1997). Therefore, the whole process of oestradiol synthesis in the follicle is a two-cell two-gonadotrophin system (Fortune & Quirk, 1988) whereby, under the direction of LH, theca cells essentially produce androgens that can be used as a substrate for oestradiol synthesis in the granulosa cells under the direction of FSH. In addition, there is evidence that the increase in oestradiol secretion positively feeds back to stimulate more androgen secretion from the theca cells (Fortune & Quirk, 1988; Roberts & Skinner, 1990; Bao & Garverick, 1998).

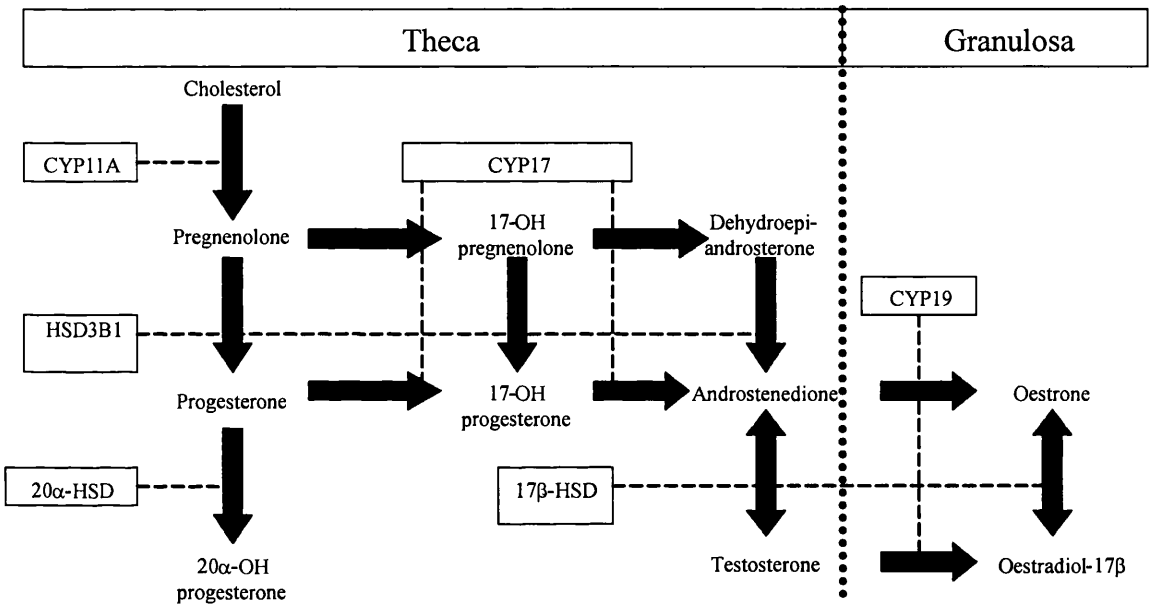


Figure 1.2 Pathways of synthesis and catabolism of steroid hormones in ovarian somatic cells. Adapted from Strauss & Penning (1999). Enzymes involved in catalysing each step are shown in boxes. (CYP11A: cytochrome P450 side chain cleavage; CYP17: cytochrome P450 17α-hydroxylase 17,20 lyase; CYP19: cytochrome P450 aromatase; HSD3B1: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; 20α-HSD: 20α-hydroxysteroid dehydrogenase).

1.4.1.6 Atresia

The vast majority of follicles (>99%) in the ewe fail to progress to a stage of terminal maturation and ovulation (Hsueh *et al*, 1994; Jolly *et al.*, 1997a, b). Instead they undergo a degenerative process known as atresia, which is characterised by sudden and widespread death of the granulosa cells (Byskov, 1978, 1979) by apoptosis. Atresia is an irreversible process that can occur at any stage of development. It is evident that atresia is not equally prevalent across all stages of follicular development, given that the greatest loss of oogonia and primary oocytes occurs during foetal life and during the prepubertal period (Hirschfield, 1989: Fotrune, 1994). The vast majority of follicles become atretic at the early antral stage of development (2-3 mm) since it is at this time that the follicles become gonadotrophin-dependent, particularly to FSH (Scaramuzzi *et al.*, 1993) and if they are not

exposed to above-baseline levels of FSH they will unavoidably die (Hirshfield & Midgley, 1978).

It also seems likely that atretic follicles may play a role in regulating ovarian function, mainly as a result of their altered steroidogenic capacity, i.e. by losing their capacity for oestrogen production (Hsueh *et al.*, 1994).

Morphologically, the pattern of atresia seems to commence with the appearance of a few apoptotic granulosa cells. Within 24h, many degenerating granulosa cells are observed throughout the granulosa layer, and within a few days, only a few remnants of the granulosa cells and oocyte can be found (Osman, 1985; Hirshfield, 1991). In pre-antral and antral follicles classical indications of atresia may include all or some of the following features: sloughing of granulosa cells into the antrum, formation of pyknotic nuclei, shortening of thecal cells, oocyte shrinkage and death, degeneration of the basement membrane, loss of the capillary network and a reduction in the proportion of mitotic bodies (Marion *et al.*, 1968; Paton & Collins, 1992).

Physiologically, the changes that occur during atresia include a reduction in granulosa cell aromatisation, with the resultant loss in aromatase activity (Huet *et al.*, 1997; Jolly *et al.*, 1997a). As a result there is a shift in the oestrogen to progesterone ratio with a general decline in oestrogen synthesis concomitant with an increase in testosterone and progesterone production (Hsueh et al., 1994).

1.4.1.6.1 Apoptosis

Apoptosis (in the ovary) is a hormonally controlled, genetically regulated process of selective cell deletion (Tilley *et al.*, 1991: Hsueh *et al.*, 1994; Elmore, 2007) occurring in a coordinated fashion that can be postponed by the action of extrinsic growth factors (Williams *et al.*, 1990). Apoptosis involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell (Thornberry & Lazebnik, 1998). Caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade (Cohen, 1997). To date, ten major caspases have been identified and broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997; Rai *et al.*, 2005). The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. To date,

research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Recent evidence has suggested that the two pathways are linked and that molecules in one pathway can influence the other (Igney & Krammer, 2002). The extrinsic and intrinsic pathways converge on the same terminal, or execution pathway (Elmore, 2007). The execution pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Cory & Adams, 2002). The extrinsic signalling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily (Locksley *et al.*, 2001). Upon ligand binding to the appropriate receptor (e.g. Fas ligand to Fas receptor), cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The relevant adaptor protein associates with procaspase-8 via dimerisation of the death effector domain. At this point, a death-inducing signalling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (Kischkel *et al.*, 1995). The intrinsic signalling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events (Elmore, 2007). The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. The control and regulation of these apoptotic mitochondrial events occurs through members of the BCL-2 family of proteins (Cory & Adams, 2002).

Intracellular effectors of apoptosis

The presence or absence of hormonal signals triggers a cascade of intracellular events in ovarian cells that ultimately induce or repress apoptosis. Several genes have been proposed to have a role in ovarian function and apoptosis, with the *BCL-2* gene family, whose genes are expressed in mammalian ovaries, the subject of much investigation (Johnson *et al.*, 1993; Flaws *et al.*, 1995; Tilly *et al.*, 1995). The *BCL-2* gene family comprises a large family of proteins, whose individual members can act in either an anti-apoptotic or pro-apoptotic manner when differentially expressed. The BCL-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. To date, a total of 25 genes have been identified in the BCL-2 family (Youle & Strasser, 2008). Essentially, they can be divided into 3 distinct subgroups: (i) anti-apoptotic proteins with multiple BCL-2 homology (BH) domains and a transmembrane region; (ii)

pro-apoptotic proteins with the same structure, but missing the BH4 domain; and (iii) pro-apoptotic ligands with only the BH3 domain (Hsu & Hsueh, 2000). The effects of BCL-2 proteins seem to be cell type-specific since, in a study using *BCL-2* gene-knockout mice, the number of oocytes and primordial follicles was dramatically reduced, but the degree of granulosa cell apoptosis was virtually unaffected (Ratts *et al.*, 1995). Conversely, mice produced following over-expression of *BCL-2* had a larger endowment of primordial follicles than normal mice, as a result of a decrease in apoptosis (Flaws *et al.*, 2001). Another member of the BCL-2 gene family that plays an important role in follicular atresia is myeliod cell leukaemia factor 1 (MCL-1) which is an anti-apoptotic protein (Sato *et al.*, 1994; Krajewski *et al.*, 1995; Hsu & Hsueh, 2000). Using the yeast 2 hybrid system MCL-1 was identified as the main ovarian anti-apoptotic BCL-2 protein (Hsu & Hsueh, 2000). *MCL-1* is expressed in the developing follicle, particularly in oocytes, granulosa cells and granulosa lutein cells and is considered to have an important follicular developmental role (Krajewski *et al.*, 1995; Hartley *et al.*, 2002). Its antagonist, *BAX*, is also expressed in granulosa cells during follicular development (Choi *et al.*, 2004). Immunohistochemical localization of BAX in the human ovary reveals abundant expression in granulosa cells of early atretic follicles, whereas BAX protein is extremely low or non-detectable in healthy or grossly-atretic follicles (Kugu *et al.*, 1998). In addition to the BCL-2 family, there are several more families of genes that mediate the effects of endocrine, autocrine and paracrine hormones and growth factors on follicle atresia. The major downstream effector of apoptosis, for both the intrinsic and extrinsic apoptotic pathways is caspase-3 (CASP3) (Matikainen *et al.*, 2001; Johnson & Bridgham, 2002), which in turn activates endogenous nucleases responsible for DNA fragmentation (Thornberry & Lazebnik, 1998). Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10) (Elmore, 2007). Studies using *CASP3* gene knockout mice determined that CASP3 is functionally required for granulosa cell apoptosis during follicular atresia (Matikainen *et al.*, 2001). Granulosa cells from healthy follicles possess, almost exclusively the inactive (unprocessed) form of CASP3, whereas granulosa cells from atretic follicles demonstrate increased concentrations of activated CASP3 (reviewed, Johnson & Bridgham, 2002; Feranil *et al.*, 2005).

Hormonal regulation of atresia

There are a multitude of factors that seem to be involved in the process of atresia, and these include various hormones (FSH, LH and GH) and growth factors (insulin, IGF-1) that act in an endocrine, autocrine, paracrine and juxtacrine manner. Prior to puberty, all of the

follicles initially recruited will become atretic due to the absence of LH pulses to support final growth, enhanced oestradiol production and ovulation (Rawlings *et al.*, 2003).

Extra-ovarian hormones

The main endocrine hormones involved in regulation of atresia are the gonadotrophins, FSH and LH. As described earlier, all ovine follicles larger than 2.5mm are gonadotrophin-dependent, as observed in studies using hypophysectomised ewes (Dufour *et al.*, 1979; Campbell *et al.*, 1999). When ewes are treated with inhibin rich bovine follicular fluid (bFF), FSH concentrations drop dramatically, yet pulsatile LH secretion is largely unaffected, resulting in the loss of all follicles that are larger than 2.5 mm (Miller *et al.*, 1979; McNeilly, 1984). However, FSH does not have a survival effect on all classes of follicles, as FSH alone does not promote the survival of pre-antral follicles (McGee *et al.*, 1997), but does enhance survival of antral follicles (Chun *et al.*, 1996).

In general, LH plays a slightly less important role initially, although it does play a key role in maintaining the health of the preovulatory follicle when FSH concentrations are declining (Scaramuzzi *et al.*, 1993; Webb *et al.*, 2003). However, LH may not always function as a survival factor since, when administered at high concentrations, this gonadotrophin can actually stimulate atresia (McNeilly *et al.*, 1992).

1.4.1.7 Ovulation

The rare follicles that escape atresia continue to grow, develop and ovulate. Late in the follicular phase, the pre-ovulatory surge of LH initiates a complex sequence of events which results in redistribution and luteinisation of granulosa and theca cells in the ovulatory follicle, inevitably giving rise to rupture of the follicle and ovulation, followed by the formation of a CL (Crisp, 1992; Richards *et al.*, 1998). Generally, there are three major changes that take place in preovulatory follicles during the process of ovulation (Richards *et al.*, 1998). Firstly, there is cytoplasmic and nuclear maturation of the oocyte. Secondly, there is disruption of cumulus cell cohesiveness among the granulosa cells and lastly the external follicular wall thins and eventually ruptures (Hafez, 1993b).

1.4.1.8 Luteinisation

Luteinisation is the key cellular response to the preovulatory gonadotrophin surge, and both granulosa and theca cell layers undergo luteinisation. There is likely to be a large number of genes involved in the process of luteinisation. However, it is most probable that it is the preovulatory surge of LH that is primarily responsible for initiating the

luteinisation process (McClellan *et al.*, 1975). Its effects are mediated via the up-regulation of LH receptors on luteal cells during luteinisation (Diekman *et al.*, 1978).

The key physiological feature of luteinisation is the switch from an oestrogenic to a progestogenic state. This is primarily achieved via two key pathways: (i) there is an up-regulation of cholesterol side-chain cleavage (CYP11A) and 3β-HSD enzymes, which convert cholesterol and pregnenolone to progesterone (Strauss & Penning, 1999); and (ii) there is a concomitant decrease in cytochrome P450 aromatase (CYP19) and cytochrome P450 17α-hydroxylase 17,20 lyase (CYP17) enzymes, which normally convert progesterone to androgens and oestrogens (Strauss & Penning, 1999). Therefore, the overall effect is a switch from high oestrogen production to high progesterone production.

1.4.2 Functional Dynamics of Follicle Growth

Describing the process of follicle growth and development based on anatomical/morphological criteria is, at best, only loosely related to the functional activity of the follicle (Crisp, 1992), as morphological criteria do not sufficiently evaluate the true physiological capacity of individual follicles (Terqui *et al.*, 1988). The terms recruitment, selection and dominance are more appropriate terms to define the various stages of follicle growth and development and are proposed as a result of the fact that follicles seem to develop in waves throughout the oestrous cycle and non-breeding season (Evans *et al.*, 2000).

1.4.2.1 Recruitment

Recruitment is the term given to the continual process in which a cohort of quiescent primordial follicles begins to mature rapidly in an environment conducive to follicular growth. This growth does not seem to be a random affair, since it seems that follicles are recruited as groups (Fortune, 1994; Webb *et al.*, 1999b; McGee & Hsueh, 2000). The mechanisms involved are unclear but may involve intra-ovarian and/or other as yet unknown factors (Findlay & Drummond, 1996). The exact number of follicles recruited in cohorts is difficult to estimate accurately as growth is slow and occurs over a prolonged period of time (Hirshfield, 1989) and because it is difficult to distinguish between growing transitional and non-growing primordial follicles (Smith *et al.*, 2008). The mechanisms regulating recruitment are somewhat unclear, but there are likely to be 3 scenarios: (i) given that quiescent follicles seem to be under some sort of constant local and/or systemic inhibitory system to remain dormant (Wandji *et al.*, 1996), initiation of growth may be due to a decrease in these inhibitory factors; (ii) initiation of growth may be a result of an increase in the actions of stimulatory factors; or (iii) initiation of growth may be a

combination of (i) and (ii). Although positive associations between FSH levels and initial recruitment have been reported (Edwards *et al.*, 1977; Wang & Greenwald, 1993), it is difficult to determine the mechanisms by which FSH exerts its effects since primordial follicles do not possess functional gonadotrophin receptors (Rannikki *et al.*, 1995; O'Shaughnessy *et al.*, 1997; Oktay *et al.*, 1997). Other intra-ovarian/oocyte-derived factors have also been proposed to have an effect on recruitment and these include anti-Mullerian hormone (AMH) (Durlinger *et al.*, 1999, 2002a, 2002b), growth differentiation factor-9 (GDF-9) (Dong *et al.*, 1996; Bodensteiner *et al.*, 1999; Vitt *et al.*, 2000b), and other members of the TGF-β superfamily (Findlay *et al.*, 2002).

1.4.2.2 Emergence

Unlike recruitment, which occurs almost continuously throughout the reproductive lifespan of the animal, selection of the species specific ovulatory quota from a cohort commences well before the onset of puberty. Selection is characterised by antral follicles (approximately 2.5mm in sheep) (Webb *et al.*, 2003) escaping the fate of atresia in response to increases in circulating FSH (McGee & Hsueh, 2000), ultimately leading to the establishment of a dominant follicle or co-dominant follicles. Using ultrasonography it has been established in sheep that between one and three antral follicles emerge or grow from a pool of small antral follicles (1–3 mm in diameter) every 4 to 5 days (Souza *et al.*, 1998; Evans *et al.*, 2000; Duggavathi *et al.*, 2004). In sheep, slight increases in plasma FSH, and consequently oestradiol, are associated with waves of follicle growth (Bartlewski *et al.*, 1999; Evans *et al.*, 2000). When elevations in the concentrations of FSH are blocked using injections of inhibin-rich bFF, this delays the first wave of follicle growth in cows (Turzillo & Fortune, 1990). Despite the fact that FSH is the predominant survival factor at this stage of follicle development, the exact cellular mechanisms by which it exerts its effects are unclear, but are likely to involve synergistic actions with intra-follicular hormones/growth factors such as IGF-1 and oestradiol (Evans *et al.*, 2000).

1.4.2.3 Selection & dominance

Following emergence, the cohort of follicles grows over the next 48-72h, after which one or two follicles are selected for further growth and become larger than the others (selection). The term dominance is given to a follicle(s) which develops rapidly, whilst other cohort follicles have their growth suppressed and undergo atresia; these are termed subordinate follicles (Fortune, 1994; Bao & Gaverick, 1998; Webb et al., 1999a). Once a follicle has attained dominance and reached its maximum size, it persists for 3-6 days before becoming atretic if the animal is in the luteal phase of the oestrous cycle (Ginther *et al.*, 1989; Knopf *et al.*, 1989). Alternatively, if luteal regression occurs during the growth

phase of the dominant follicle, the follicle continues to remain dominant over the entire follicular phase and eventually ovulates (Kastelic *et al.*, 1990).

It is somewhat unclear how one or two follicles are selected to be dominant from a pool of similarly sized cohort follicles. In the bovine, the main morphological difference that is observed when follicles are selected for dominance is follicle diameter (Ginther *et al.*, 1996; Fortune *et al.*, 2001). Follicle diameter increases very rapidly in dominant follicles relative to subordinate follicles, whose growth rates reduce dramatically. The actual point at which selection of a dominant follicle occurs is difficult to ascertain, since the first signs of follicle deviation are likely to be biochemical/molecular and not just a difference in size (Fortune *et al.*, 2001). However, the follicle that is eventually selected as being dominant is likely to have attained this position by virtue of having an increased capacity to respond to small increases in FSH and by producing larger quantities of oestradiol (Badinga *et al.*, 1992; Bodensteiner *et al.*, 1996; Evans & Fortune, 1997). In addition, the dominant follicles selected are those that have acquired more LH receptors, particularly on their granulosa cells, and this facilitates oestradiol synthesis in response to LH as well as FSH (Fortune, 1994; Ginther *et al.*, 1996). Indeed, various studies have demonstrated that dominant follicles have increased levels of mRNAs encoding various steroidogenic enzymes (e.g. *CYP19A1*, *CYP17*, *CYP11A*, *HSD3B1*, *StAR*) in addition to gonadotrophin receptors (Fortune, 1994; Xu *et al.*, 1995; Bao *et al.*, 1997; Bao & Gaverick, 1998; Webb *et al.*, 1999a) hence their ability for increased steroid synthesis. Recent molecular evidence in the bovine shows that the dominant follicle continues to acquire more LH receptors together with a reduction in FSH receptors (Mihm *et al.*, 2006) and, as a result within the dominant follicle, there is a transfer from FSH to LH dependence.

It is uncertain how one follicle continues to grow and is dominant, while others fail to. The most likely hypothesis is that the dominant follicle produces large amounts of oestradiol and inhibin which feedback at the hypothalamo-hypophyseal level to reduce FSH secretion (Ginther *et al.*, 2000a). Hence, FSH concentrations drop to a level that is inadequate for subordinate follicles to grow and, thus, they become atretic. However the dominant follicle has reached a level of growth and differentiation that facilitates it remaining healthy and growing, even in the face of lower concentrations of FSH (Zeleznik & Kubik, 1986; Ginther *et al.*, 1999, 2000b).

1.4.3 Regulation of Follicle Growth & Development

The process of follicle growth and development is regulated by many extra- and intra-ovarian hormones and growth factors.

1.4.3.1 Extra-ovarian regulation

It has been well established that the gonadotrophins, FSH and LH, are the primary endocrine hormones that regulate follicle growth and development. Their role will be discussed, as will the role that other extra-ovarian hormones play in regulating the process of follicular growth and development.

Follicle-Stimulating Hormone

As stated previously, FSH plays the major role in growth and maturation of ovarian antral follicles (Phillips *et al.*, 1994). Despite the suggestion that FSH may be involved in the initiation of primordial follicle growth (Peters, 1979), in sheep, as in many other mammals, there does not seem to be an essential requirement for gonadotrophins in the early stages of follicle growth since studies have shown that pre-antral (Dufour *et al.*, 1979) and antral follicles up to 2.5mm in diameter are still evident in ewes following hypophysectomy (Driancourt *et al.*, 1987; McNatty *et al.*, 1990). Granulosa cells acquire FSH-specific receptors during the pre-antral stage of their development (Liu *et al.*, 1998). Specifically, FSH receptor mRNA has been detected in follicles with only 1-2 layers of granulosa cells (Tisdall *et al.*, 1995); if follicles are to progress to the antral stage of development the granulosa cells must competently respond to FSH stimulation (Zeleznik & Hillier, 1984). If they fail to achieve this, their fate is likely to be an atretic one since studies have shown that atresia is accompanied by a decrease in levels of FSH receptors and their mRNAs in granulosa cells (Abdennebi *et al.*, 1999).

The actions of FSH are not solitary and can be synergistic, as evidenced by a wealth of information illustrating the inter-play between FSH and various intra-ovarian growth factors, such as the insulin-like growth factors (IGFs), oestradiol and inhibin (Campbell & Webb, 1995; Campbell & Baird, 2001).

At the granulosa cell level, in addition to granulosa cell proliferation, FSH plays an essential role in granulosa cell differentiation (McNatty *et al.*, 1992). Firstly, FSH induces aromatase activity in healthy large antral follicles (>3mm in diameter in sheep) (McNatty *et al.*, 1992; Duggavathi *et al.*, 2006), secondly in conjunction with oestradiol, FSH has been shown to stimulate the development of LH receptors on granulosa cells (Erickson *et*

al., 1982; Lapolt *et al.*, 1990; Kanzaki *et al.*, 1994), and finally FSH stimulates the production of oestradiol and progesterone from the granulosa cells of preovulatory follicles (Campbell *et al.*, 2003; Duggavathi *et al.*, 2006).

Luteinising Hormone

Whilst FSH clearly has a primary role in regulating the growth of follicles and antrum formation, as stated earlier LH plays its primary role in follicle development and growth during terminal maturation of the ovulatory follicle(s) (Campbell *et al.*, 1999).

The pattern of LH receptor appearance is similar to that of FSH receptors in that LH receptors can also be present in follicles from an early stage of development. In particular they are localised to the theca cells during the early stages of follicular development. However, during the later stages of growth, granulosa cells have been shown to express LH receptors (Richards *et al.*, 1987; Yuan *et al.*, 1996; Abdennebi *et al.*, 1999), implicating LH in the terminal maturation process (McLeod *et al.*, 1982). LH has similar actions to FSH in that it is mitogenic and strongly differentiative. LH has been shown to increase the proliferation of thecal cells *in vitro*, and studies also indicate that it increases LH receptor numbers (Magoffin & Weitsman, 1994) as well as androgen and progesterone production (Campbell *et al.*, 1995; Armstrong *et al.*, 1996). One of the ways in which LH stimulates granulosa cell differentiation is by stimulating aromatase activity (Scaramuzzi *et al.*, 1993).

1.4.3.2 Intra-ovarian regulation

The gonadotrophins play key roles in the regulation of follicle growth and development. However, it has become evident that there are a number of other hormones/growth factors that seem to act in an autocrine and/or paracrine manner that also affect the processes of follicular growth and development. The roles of growth factors in follicular development and survival are dependent on gonadotrophin status and differentiation state of the follicle (Webb *et al.*, 2003). Locally produced intra-follicular factors have been implicated in the processes of follicular proliferation and differentiation in the sheep, as in other mammalian species. The most important of these seem to be inhibin, activin, follistatin and members of several growth factor superfamilies. Many of these factors are produced in various cell compartments within the follicle, namely the granulosa, theca and oocyte.

Oocyte-derived factors

In recent years it has emerged that the oocyte can also regulate granulosa cell proliferation and differentiation (Eppig, 2001). Two oocyte-derived growth factors, which are members of the TGF-β superfamily seem to play key roles in development; (i) growth differentiation factor-9 (*GDF-9*); and (ii) bone morphogenetic protein-15 (*BMP-15*), which is also known as *GDF-9B*. *GDF-9* is produced by both sheep and cattle oocytes throughout follicle development and ovulation (Bodensteiner *et al.*, 1999). The importance of *GDF-9* for early follicle development has been illustrated in mice, since follicles in null-mutant mice for *GDF-9* fail to develop beyond the primary follicle stage due to a complete failure in the development of the somatic cells (Dong *et al.*, 1996; Carabatsos *et al.*, 1998; Elvin *et al.*, 1999b). In addition, in *GDF-9* null-mutant mice, there appears to be an increase in the expression of the *KIT1* gene by granulosa cells (Elvin *et al.*, 1999b), which encodes kit ligand (KL), concomitant with increases in the concentrations of circulating FSH (Dong *et al.*, 1996). Thus, *GDF-9* seems to be an important inhibitor of KL production, KL being an important factor in promoting oocyte growth and development (Packer *et al.*, 1994; Reynaud *et al.*, 2000). Therefore, GDF-9 may act as an autocrine regulator of oocyte growth and development by inhibiting KL-stimulated growth of oocytes. BMP-15 protein is also produced by oocytes and the *BMP-15* gene was first shown to have a role in ovarian function in studies using the Inverdale and Hanna breeds of sheep. These sheep carry naturally occurring X-linked mutations that cause an increase in ovulation rate in heterozygotes ($FecX^{I}/FecX^{+}$) and ovarian failure in homozygotes ($FecX^{I}/FecX^{I}$) (Davis *et al.*, 1991; 1992; Braw-Tal *et al.*, 1993; Smith *et al.*, 1997; Galloway *et al.*, 2000), which is similar to that observed in *GDF-9* null-mutant mice. Thus *BMP-15*, like *GDF-9* is essential for early follicular development in sheep, specifically the primary to secondary transition. The exact mechanism by which follicular development is increased in the heterozygous animal is not yet fully apparent, and there seems to be little or no difference in gonadotrophin levels in these mutant sheep.

Both *GDF-9* and *BMP-15* are mitogenic, promoting the proliferation of granulosa cells from small antral follicles (Hayashi *et al.*, 1999; Otsuka *et al.*, 2000; Vitt *et al.*, 2000a). In terms of differentiative properties, *GDF-9* produced by fully grown oocytes can suppress the expression of LHR (Elvin *et al.*, 1999a; Joyce *et al.*, 2000), and stimulate progesterone production from granulosa cells (Elvin *et al.*, 1999a). Conversely, *BMP-15* has been shown to suppress FSH-induced production of progesterone by granulosa cells (Otsuka *et al.*, 2000).

Inhibins, activins and follistatin

The regulatory roles of inhibin, activin and follistatin in controlling reproduction at the endocrine level have been highlighted earlier. However, it has become evident that these peptides also execute an intra-ovarian function. Inhibin, activin and follistatin have opposing actions in the process of steroidogenesis, suggesting they are part of a complex intra-ovarian regulatory system that has yet to be fully elucidated. Moreover, because they can regulate the amount of androgen substrate available for oestradiol synthesis by follicles, these factors are also likely to be important in the processes of selection and dominance.

Inhibin is produced by the granulosa cells of follicles, although in primates it is also produced by luteal cells (Findlay, 1993). It seems that inhibin may play a role in regulating steroidogenesis in both theca and granulosa cells. Inhibin has been shown to suppress FSH-induced aromatase activity in cultured rat (Ying *et al.*, 1986) and primate (Miro & Hillier, 1992) granulosa cells. In cattle (Wrathall & Knight, 1995), human (Hillier, 1991) and rat (Hsueh *et al.*, 1987) theca cells, inhibin has been found to increase LH-induced androgen production. Inhibin, therefore, seems to be involved in ensuring that there are adequate levels of androgen produced to facilitate increasing oestradiol synthesis in preovulatory follicles.

Activin is important for follicle development and has been shown to induce proliferation of granulosa cells in various sized follicles *in vitro* (Li *et al.*, 1995; Miro & Hillier, 1996). This is supported by a study utilising knockout mice lacking the activin type IIB receptor, where follicle development was arrested at the early antral stage, although this may also be in part due to the fact that FSH concentrations are also reduced in these animals (Nishimori & Matzuk, 1996). Activin has also been shown to increase (i) FSH receptor expression (Xiao *et al.*, 1992b); (ii) FSH-induced aromatase (Hillier & Miro, 1993); (iii) oestradiol production (Hutchinson *et al.*, 1987) and (iv) oocyte maturation (Alak *et al.*, 1998; Sidis *et al.*, 1998). In addition, activin has also been associated with a delay in the onset of atresia and luteinisation (Hutchinson *et al.*, 1987), and a decrease in LH-induced androgen production (Wrathall & Knight, 1995).

An intra-ovarian role for follistatin was illustrated with the finding that its presence can reverse the inhibitory effects of activin on both LH- and oestradiol-induced androgen secretion in theca cells (Wrathall & Knight, 1995). Given that follistatin is not produced in any great quantities by relatively undifferentiated granulosa cells (Shimasaki *et al.*, 1989;

Nakatani *et al.*, 1991) – cells that produce large amounts of activin, and have many activin binding sites present (Findlay *et al.*, 2000) – this is likely to be one of the reasons activin is 'free' to promote FSH receptor expression in small follicles. In addition, follistatin may be involved in promoting follicle atresia or luteinisation, given that studies in rats have indicated that follistatin can suppress aromatase activity and inhibin production, concomitant with increasing progesterone production (Xiao *et al.*, 1992a).

Oestrogen

It is well established that steroids perform an important feedback role to regulate gonadotrophin secretion, but it has become apparent that oestrogen in particular may be a key intra-ovarian modulator of follicle growth and development (Schams & Berisha, 2002). The principal oestrogen is oestradiol-17β which can bind to two forms of receptor, (ERα and ERβ) (Kuiper et al., 1996; Drummond et al., 1999). ERα and ERβ exhibit species-/tissue-/cell-specific localisation and levels of expression (Drummond & Fuller, 2010). The beta form of the oestrogen receptor is primarily present in the ovary (granulosa cells, theca cells, corpora lutea (CL), and oocyte) (Drummond *et al.*, 1999b; Pelletier *et al.*, 2000; Juengel *et al.*, 2006), while ERα, although also localised to the ovary (granulosa and theca cells) has been found predominantly in testis, epididymis, mammary gland, brain (pituitary gland), adipose, bone, heart, and uterus (Drummond *et al.*, 1999b; Pelletier *et al.*, 2000; Taylor & Al-Azzawi, 2000; Juengel *et al.*, 2006; Weiser *et al.*, 2008). Information on the expression of the respective ER mRNAs and proteins in granulosa cells of different follicle sizes is limited (Drummond & Fuller, 2010). *In situ* hybridisation and RT-PCR studies in the rat indicate that there is more ERβ than ERα mRNA in the ovary (Drummond *et al.*, 1999b) and mRNAs for ERα and ERβ are present in granulosa cells of follicles with at most two to three layers of granulosa cells (Drummond *et al.*, 1996; Drummond *et al.*, 1999b; Juengel *et al.*, 2006).

Oestrogen has marked effects on somatic cell proliferation and differentiation. It has been demonstrated that oestrogen is a potent mitogenic agent, stimulating proliferation of granulosa cells, and ultimately it increases the number and size of ovarian follicles *in vivo* and *in vitro* (Gore-Langton & Daniel, 1990; Hulshof *et al.*, 1995). In terms of oestrogen's properties to differentiate cells, it induces the appearance of receptors for FSH and LH (Drummond & Findlay, 1999; Bao et al., 2000). In addition, studies in rats highlight that, as follicles develop to medium size, the number of oestrogen receptors increases, and this is accompanied by increases in the expression of aromatase in granulosa cells (Bao *et al.*, 2000), further signifying a role for oestrogen in follicle growth and development.

1.5 The Androgenised Ewe Model

Animal models that exhibit altered/reduced reproductive ability, specifically disrupted or arrested follicular development, provide valuable resources to investigate the intra-follicular environment that is required for normal follicular growth and development. One such model is the prenatally androgen treated ewe. This model is also very useful for determining the effects of environment on reproductive physiology. In this model, pregnant ewes are injected with 100mg of either testosterone propionate (TP) or the non aromatisable androgen, 5α-dihydrotestosterone (DHT) twice weekly during a specific window of *in utero* development (days 30-90 - term = 147 days). It is during this period that the reproductive axis and external genitalia become sexually differentiated (Clarke *et al.*, 1976). Numerous studies have found that excess prenatal testosterone treatment during this period leads to a range of reproductive, growth and metabolic disruptions as well as abnormalities in the external genitalia and reproductive behaviours. Studies using both TP and DHT models have focused mainly on two breeds of sheep; the Suffolk and the Poll Dorset. These breeds have similar length breeding seasons (September/October-January), are of similar adult weights and normally produce about 1.5 offspring per pregnancy. Despite these similarities differences between the two breeds in the degree of reproductive disruption caused by foetal androgen exposure have been observed and are highlighted when present. The breed used in the studies described in this thesis is the Poll Dorset.

1.5.1 Disruption of reproductive function

1.5.1.1 Reproductive cyclicity

Exposure of the female lamb to prenatal TP results in a suite of adult reproductive disorders (Clarke *et al.*, 1977; Wood & Foster, 1998; West *et al.*, 2001; Sharma *et al.*, 2002; Birch *et al.*, 2003; Savabieasfahani *et al.*, 2005; Steckler *et al.*, 2005; Unsworth *et al.*, 2005; Steckler *et al.*, 2007a) ultimately leading to reproductive failure (Clarke *et al.*, 1977; Birch *et al.*, 2003). Early studies by Clarke et al. (1977) showed that Finnish Landrace/Dorset Horn cross ewes, whose mothers had been implanted with 1g testosterone for different periods of pregnancy, showed marked disruption of ovulatory cycles. Specifically, the percentage of ewes ovulating having been androgenised between days 30 to 80 or 50 to 100 was 57% and 77%, respectively. Importantly, none of these ewes had regular cycles during the period of observation, confirming the idea that androgenised ewes either do not ovulate or do so at erratic intervals. In contrast, ewes exposed to androgen between days 90 and 140 of foetal development had normal regular cycles in adulthood (Birch *et al.*, 2003; Steckler *et al.*, 2007b). The 'critical period' and dose of androgen required to disrupt cycles in the Suffolk ewe was further refined by scientists in

Doug Foster's group at The University of Michigan (Wood & Foster, 1998) and the timing and dose of androgen used in our studies have been informed by this extensive work. In recent years two separate groups have revealed that the time of the first reproductive cycle in androgenised Dorset and Suffolk ewes is similar to that of control animals (Sharma *et al.*, 2002; Manikkam *et al.*, 2006). However, cycles in the androgenised animals show disruptions in that only 72% of the Dorset ewes had cycles in the first breeding season and none in the second breeding season (Birch *et al.*, 2003). Similar studies in the Suffolk ewe (Sharma *et al.*, 2002) revealed a less extreme phenotype with the duration of the first breeding season and the number of reproductive cycles being similar between controls and androgenised animals. It should, however, be noted that in the Dorset breed of sheep the complete absence of cyclicity in the second breeding season found in an earlier study (Birch *et al.*, 2003) was not evident in a later study (Unsworth *et al.*, 2005). Although the number of cycles was substantially reduced, changes in progesterone concentrations that meet the criteria for a cycle (see Materials and Methods) were evident. Studies performed for over a decade have shown that the number of normal, regular cycles in the androgenised Dorset ewe varies from year to year (Jane Robinson; personal communication) suggesting that other environmental, genetic or experimental factors may also play a role. Additionally, a group of animals that were exposed to TP from day 60-90 of gestation (the second half of the 'critical period') had fewer cycles than the controls in their first breeding season (86%) which was further reduced in the second season (Birch *et al.*, 2003). This was not observed in the study by Sharma et al., (2002). The severity of reproductive defects and the timing of onset of reproductive perturbation programmed by prenatal TP excess are highly variable between breeds and within individuals of the same breed which highlights the interaction between genetics and environment.

1.5.1.2 Steroid Feedback Mechanisms

As detailed earlier, for proper patterning of gonadotrophin secretion during development and cyclicity both stimulatory and inhibitory neuroendocrine steroid feedback mechanisms must be effective. In order to study these feedback mechanisms ewes of both the Dorset and Suffolk breeds have been ovariectomised soon after birth and a one centimetre long Silastic implant of oestradiol is implanted subcutaneously to provide an unvarying physiological concentration of the steroid (1-2pg/ml). Using this ovariectomised steroid replaced model (OVX + E) extensive studies have demonstrated that prenatal TP treatment reduces hypothalamic sensitivity to all three major feedback mechanisms involved in the control of the cyclic changes in GnRH/gonadotrophin secretion, namely (1) oestradiol negative feedback, (2) oestradiol positive feedback (Wood & Foster, 1998; Sharma *et al.*,

2002; Sarma *et al.*, 2005; Unsworth *et al.*, 2005) and (3) progesterone negative feedback (Robinson *et al.*, 1999). Oestradiol negative feedback is the predominant feedback system operational before puberty, with a reduction in the sensitivity to this feedback mechanism responsible for the pubertal rise in GnRH and LH release (Foster, 1994). Investigations using the OVX + E ewe found that exposure to prenatal TP advances the time of the pubertal rise in LH from about 30 weeks of age to about 10 weeks (Wood & Foster, 1998). This is similar to the time of the LH rise in ram lambs that have been gonadectomised and implanted with a small implant of oestradiol (7-8 weeks). Studies using ovary-intact androgenised sheep, found females to be hypergonadotrophic (LH only) and exhibit reduced sensitivity to oestradiol negative feedback (Sarma *et al.*, 2005). Similar responses have been observed in the DHT exposed animals showing that testosterone does not need to be aromatised to oestrogen to have these actions (Wood & Foster, 1998).

In addition to the disruption of oestradiol negative feedback, prenatal TP treatment also alters oestradiol positive feedback (Wood & Foster, 1998; Sharma *et al.*, 2002; Birch *et al.*, 2003; Unsworth *et al.*, 2005) which is necessary for the generation of the preovulatory GnRH/LH surge (Moenter *et al.*, 1991). Specifically, the prenatally TP-treated OVX + E ewe of both breeds is unable to respond to follicular-phase concentrations of oestrogen with a preovulatory-like surge of GnRH/LH (Wood *et al.*, 1995; Herbosa *et al.*, 1996). We know that the masculinisation of oestrogen positive feedback is brought about by the oestrogenic actions of TP because prenatal exposure to DHT does not disrupt the ability of oestrogen to trigger the GnRH surge (Wood & Foster, 1998). The ovary-intact Dorset ewe (like the OVX + E Dorset) is unable to respond to exogenous oestrogen with a LH surge, either before puberty or just before the start of the second breeding season (Unsworth *et al.*, 2005). In contrast LH surges were present, but delayed and dampened in the ovary intact Suffolk ewe (Sharma et al 2002). The difference may lie in the breed of sheep used (Suffolk vs. Dorset) as, although these breeds are of similar adult weight, their body composition may be sufficiently different to affect the distribution and metabolism of the administered steroid and, thus, the concentration delivered to the foetus. Reasons for the difference in the ability to respond to oestradiol with an LH surge in the ovary-intact versus the OVX + E Suffolk ewe are discussed later.

The final feedback mechanism altered in prenatally TP-treated animals is the action of progesterone inhibition, important for suppression of the release of GnRH during the luteal phase of the oestrous cycle and the blockade of ovulation (Karsch, 1987). This was tested in OVX + E Dorset ewes using a low physiological concentration of exogenous

progesterone provided by a single intravaginal CIDR device (2-3 ng/ml). Prenatal TP females manifested reduced responsiveness to progesterone negative feedback in a similar manner to males (Robinson *et al.*, 1999). This was observed both in the presence and absence of oestrogen (which induces receptors for progesterone) and at the time of the breeding and anoestrous seasons.

Associated with these neuroendocrine feedback abnormalities is an alteration in gonadotrophin secretion manifested as hypersecretion of LH but not FSH (Sharma *et al.*, 2002; Sarma *et al.*, 2005). Hypersecretion of LH associated with reproductive abnormalities is also evident following prenatal exposure to excess testosterone in female rhesus monkeys (Abbott *et al.*, 2005) (see section 1.5), mice (Sullivan & Moenter, 2004), and rats (Foecking *et al.*, 2005) and it is also seen in women with polycystic ovary syndrome (Katz & Carr, 1976; Rebar *et al.*, 1976). A relatively recent study investigating the mechanisms that mediate hypersecretion of LH determined that excess prenatal TP treatment, by its androgenic action only, amplified the GnRH induced LH response, leading to LH hypersecretion (Manikkam *et al.*, 2008). It is thought that this programming involves developmental changes in expression of pituitary genes responsible for LH synthesis and release.

1.5.1.3 Ovarian Defects

In addition to reproductive neuroendocrine disruptions, excess prenatal TP treatment leads to several ovarian defects in sheep (West *et al.*, 2001; Steckler *et al.*, 2005; Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). The first study to investigate the ovarian histology in prenatal TP-treated sheep found that ovaries of 5 week old Suffolk female lambs were heavier/larger and observed a mutlifollicular morphology, with many large antral follicles visible (West *et al.*, 2001). This had also been noted in 3 week old Dorset lambs (Wood *et al.*, 2000). Furthermore, more recent studies have determined that both follicle numbers and ovarian volume are increased in prenatally TP-treated ewes (Manikkam *et al.*, 2006). A possible explanation for the multifolliculate condition observed in the androgenised ewe is that these antral follicles fail to regress (undergo atresia). This hypothesis was tested by Manikkam et al., (2006) who determined that the persistence of antral follicles was significantly longer in prenatally TP-treated ewes. However, larger ovaries, multifollicular morphology and increased numbers of primary, preantral, and antral follicles (growing follicles) at 10 months of age (Steckler *et al.*, 2005) are only observed when sheep have been prenatally treated with TP and not DHT, demonstrating that these ovarian features are

mediated through the action of oestrogen and not androgens (Steckler *et al.*, 2005; Steckler *et al.*, 2007a).

Additional studies in the Suffolk breed of sheep have found decreased numbers of primordial and increased numbers of growing follicles in prenatal testosterone and DHT-treated foetuses on day 140 of gestation (D140), suggesting enhanced follicle recruitment from the primordial pool (Steckler *et al.*, 2005; Smith *et al.*, 2008). However, no differences in numbers of oocytes and follicles were determined in either prenatal testosterone or DHT-treated foetuses on day 90 (D90) of gestation (Smith *et al.*, 2008), suggesting that prenatal TP and DHT excess does not affect germ cell development and primordial follicle formation. The absence of differences in germ cells in both prenatal testosterone and DHT-treated foetuses on D90 of gestation provides evidence that any difference in ovarian reserve seen at a later time point are likely to be as a result of increased follicular recruitment or follicle persistence and not differences in initial ovarian reserve. In 10 month old sheep, increased numbers of growing follicles and reduced numbers of primordial follicles were found in prenatally TP-treated females but not in DHT-treated females (Smith *et al.*, 2008). Studies using cortical biopsies from ovaries of 8 month old Dorset sheep, while not controlled for cycle stage as in the Steckler 2005 study, provided further support in favour of enhanced recruitment (Forsdike *et al.*, 2007).

The mechanisms by which prenatal TP treatment programs increased ovarian size and multifollicular ovaries are not clear. On the one hand increased number of antral follicles may contribute towards increased ovarian size. Alternatively, this may be as a result of increased stromal volume, a feature which has not yet been explored in prenatal TP-treated females.

A study into luteal function in the Suffolk model demonstrated prenatal TP excess reduced the number of corpora lutea in those animals that cycled (Manikkam *et al.*, 2006). This finding suggests that these large antral follicles are unable to luteinise in the proper manner and instead continue to proliferate and grow (failure to undergo atresia).

1.5.1.4 Gonadal and Genital Development

Gonadal steroids mediate sexual differentiation of the reproductive phenotype. Early studies in rabbits initiated the concept that testosterone acting systemically and anti-mullerian hormone acting locally, directs the differentiation of the male internal genitalia (Jost *et al.*, 1973). In genetic males (XY), the gonads differentiate into testes. The Wolffian

duct then proliferates in the presence of testosterone into the vasa deferentia and seminal vesicles. In females (XX), the primordial gonads differentiate into ovaries. Then, the Mullerian duct develops (as Mullerian inhibiting factor is absent) to give rise to the uterus, fallopian tube and vagina. The bipotential external genitalia in the presence of DHT are masculinised to form the penis and scrotum. Therefore, external appearance of male or female organs depends on the presence or absence of testosterone during a critical period of development.

Prenatal TP exposure has a virilising effect on the external genitalia of female lambs. TP-treated lambs have genitalia that are similar to males, in that they exhibit a penis and scrotal tissue, but with no testicular tissue. Internally, the ovaries are located in the correct anatomical position (Wood & Foster, 1998) and exhibit a blind-ending uterus but no vagina (Unsworth *et al.*, 2005). The degree of masculinisation of the external genitalia in the female has been found to be proportional to the concentration amount of TP exposure (Wood & Foster, 1998). Using a 200mg weekly dose, masculinisation was maximal, with development of a penis and an empty scrotum. Partial masculinisation occurred with an 80mg dose, with a split scrotum and urethral opening between the two empty scrotal folds at an intermediate distance between those of normal males and females. Minimal masculinisation was found with a 32mg dose, with normal placement of the urethral opening. Timing was also found to be important with androgens present between days 30 and 50 of gestation causing genital abnormalities but not between days 65 and 85 of pregnancy (Foster *et al.*, 2002). Alteration of the female reproductive phenotype towards male phenotypic features also occurs in humans from prenatal androgen exposure. Congenital adrenal hyperplasia, the commonest cause of genital ambiguity of the external genitalia at birth in female children is invariably as a result of 21-hydroxylase deficiency (Hughes, 1998).

1.5.2 Foetal and Postnatal growth

Steroid hormones have effects on normal growth of body tissues in concert with growth hormone effects. Studies in sheep have shown alteration of normal foetal growth can be programmed by inappropriate foetal steroid exposure. TP-treated Suffolk sheep demonstrate foetal growth retardation and reduced birth weight and height (Manikkam *et al.*, 2004; Steckler *et al.*, 2005). Additionally, prenatally TP-treated ewes, but not the males, exhibit catch-up growth between 2-4 months of their birth (Manikkam *et al.*, 2004). Unpublished studies in the Dorset ewe have shown that both TP and DHT animals have significantly lighter body weights at birth but both exhibit catch up growth about the time

of puberty. The TP ewes then continue to grow compared to controls and DHT ewes and are significantly heavier by about 10 months of age (Robinson JE, Hastie PM, and Evans, unpublished data). Studies in rats have also found a significant reduction in pup birth weight in both sexes when foetuses were exposed to TP (Wolf *et al.*, 2002). Reduced birth weight (intrauterine growth retardation) and postnatal catch-up growth are both viewed as risk factors for adult onset diseases like metabolic syndrome (Ong & Dunger, 2002).

1.5.3 Prenatal androgen excess causes metabolic problems

It is well known that sex steroids influence insulin sensitivity (insulin sensitivity refers to the actions of insulin on glucose), with androgenic contraceptives taken orally by women leading to insulin resistance (Godsland *et al.*, 1992). Prenatal exposure to TP in sheep leads to elevated insulin concentrations in fasted adult sheep (hyperinsulinaemia) as well as a significantly increased release of insulin in response to an iv infusion of glucose (insulin resistance). This has been reported in both the Dorset and Suffolk breed (DeHaan *et al.*, 1987; Rosser *et al.*, 2003).

1.6 Polycystic ovary syndrome (PCOS)

PCOS is the most common endocrine disorder in women of reproductive age, affecting approximately 5-10% of women of reproductive age in the developed world (Dumesic *et al.*, 2007; Franks *et al.*, 2008). PCOS is the major cause of anovulatory infertility, menstrual disturbances and hirsutism (excessive hairiness). Clinical and biochemical features of PCOS are typically heterogeneous, but the distinctive multifollicular ovarian morphology is a characteristic feature of the syndrome (Tsilchorozidou *et al.*, 2004; Franks *et al.*, 2006; Franks *et al.*, 2008). The cause of the syndrome remains uncertain, although there is evidence that genetics have an important role, with the disease thought to be as a result of an interaction of a small number of key genes with environmental and in particular nutritional factors (Franks *et al.*, 1997).While its peripubertal onset and familial clustering suggest a heritable etiology for PCOS, several candidate genes, including those regulating insulin action, androgen biosynthesis and gonadal function, have failed to fully explain its prevalence (Dumesic *et al.*, 2007). One of the possible causes of PCOS is prenatal androgen excess and recent studies have provided additional evidence in support of this (Xita & Tsatsoulis, 2006). Ovarian hyperandrogenism is the cardinal feature of PCOS, with *in vitro* studies of PCOS theca cells showing intrinsically increased androgen biosynthesis and augmented expression of several steroidogenic enzymes, including cytochrome P450 cholesterol side chain cleavage (CYP11A1), 17α-hydroxylase/17–20 lyase (CYP17A1) and 3ß-hydroxysteroid dehydrogenase (HSD3B1) (Nelson *et al.*, 1999;

Nelson *et al.*, 2001). As highlighted earlier, many of the neuroendocrine, metabolic and ovarian disruptions of the prenatally TP-treated ewe are similar to those seen in women with PCOS, thus the androgenised model has evolved as an alternative for understanding the etiology of polycystic ovary syndrome.

The characteristic morphological feature of polycystic ovaries in anovulatory women is an accumulation of antral follicles in the range of 2-8mm in diameter (Franks *et al.*, 2008). There is conflicting evidence in support of enhanced follicular recruitment in PCOS patients. A reciprocal increase and decrease in the proportions of primary and primordial follicles, respectively, in ovaries of women with PCOS was reported in one study (Webber *et al.*, 2003), although other studies can be interpreted to refute these findings (Hughesdon, 1982; Maciel *et al.*, 2004). Excess postnatal weight gain amplifies reproductive disruptions caused by TP excess, as fewer obese TP-treated Suffolk ewes are able to generate an LH surge (Steckler *et al.*, 2009), an observation that is also seen in women with PCOS (Dumesic *et al.*, 2007).

In addition to the prenatally androgenised ewe model, there are several other models of PCOS, most notably that of the rhesus monkey (Dumesic *et al.*, 1997; Abbott *et al.*, 2008; Dumesic *et al.*, 2009). Prenatal TP-treated monkeys, like PCOS patients, manifest anovulatory infertility (Abbott *et al.*, 2004), adiposity-dependent compensatory hyperinsulinemia (Abbott *et al.*, 2005; Recabarren *et al.*, 2005), hypergonadotropism (Dumesic *et al.*, 1997), neuroendocrine feedback defects (Steiner *et al.*, 1976; Dumesic *et al.*, 2002), functional hyperandrogenism (Eisner *et al.*, 2002; Zhou *et al.*, 2005) and polycystic ovaries (Dumesic *et al.*, 2002). In addition, like the androgenised ewe model, prenatal androgenisation in the rhesus monkey accelerates follicle differentiation and diminishes the ovarian reserve (Dumesic *et al.*, 2009). However, interestingly, the prenatally TP-treated rhesus monkey does not show intrauterine foetal growth retardation or post natal catch-up growth (Abbott *et al.*, 2008).

1.7 Introduction to Experimental Approaches

In summary, follicle growth and development is a complex system which is regulated by a variety of extra- and intra-ovarian hormones. The prenatally androgenised ewe provides an excellent model to investigate follicular growth and development, specifically at the terminal follicle stage. Additionally, the use of the prenatally androgenised ewe facilitates further characterisation at the individual follicle level of polycystic ovary syndrome due to the parallels between the syndrome and the animal model. Furthermore, to the best of our

knowledge, there has been no investigation in identifying abnormalities in large persistent antral follicles observed on ovaries from ewes prenatally treated with either TP or DHT. Therefore, this study provides novel information into the abnormalities within large antral follicles from prenatally androgenised ewes. Lastly, results from the study will contribute to our understanding of prenatal programming of adult ovarian function.

The central aim of the experimental work undertaken in this thesis has been to identify abnormalities in large antral follicles from prenatally androgenised ewes, and where/when abnormalities were determined, to further characterise underlying cellular changes in these follicles. Further characterization centred on determining (1) steroid hormone levels in the follicular fluid of abnormally large follicles (Chapter **3**), (2) steroidogenic capability in smaller antral follicles (Chapter **4**) and (3) differentially expressed genes controlling steroidogenesis, proliferation, atresia and differentiation in antral follicles in granulosa cells from abnormally large follicles (Chapter **5**). Further to this central aim, two secondary aims comprised (1) assessing health status in early antral follicles (Chapter **4**) and (2) determining peripheral steroid concentrations in prenatally androgenised ewes (Chapter **3**). The final aim comprised a functional study, determining if granulosa cells are intrinsically different between androgenised and control ewes in respect to unstimulated or gonadotrophin-dependent steroid production (Chapter **6**).

Four experimental chapters are reported in this thesis (chapters **3** to **6**) and a description of Materials and Methods common to several experimental chapters is contained in General Materials and Methods (Chapter **2**). Within each experimental chapter a brief discussion of the findings is given. A General Discussion (Chapter **7**) is used to collate the findings from all of the experiments.

CHAPTER 2: GENERAL MATERIALS AND METHODS

This chapter contains a detailed description of the Materials and Methods used in each experiment.

2.1 ANIMAL HUSBANDRY

All animal procedures used in the study were approved by the Faculty's Ethics and Welfare Committee and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 (Project number PPL_60/3485; 'Prenatal programming of reproductive function'). Sheep were maintained under normal husbandry conditions at the University Farm, Cochno Road, Glasgow, UK. In winter months they were housed indoors and in the summer months they were on pasture. Lambs were obtained from a breeding flock of Poll Dorset ewes which has been maintained at the University Farm since 2003.

2.2 THE *IN UTERO* ANDROGENISED EWE MODEL

Experiments were conducted using control ewes (C) and those that were exposed to androgens during foetal development (androgenised ewes). Androgen treatment involved foetal exposure to testosterone propionate (TP) or the non aromatisable androgen, 5α-Dihydrotestosterone (DHT) employing an identical method to that previously described (Steckler *et al.*, 2005; Unsworth *et al.*, 2005). Briefly, the reproductive cycles of ewes from the breeding Dorset flock were synchronized using an intravaginal CIDR device (Inter Ag, NZ), and animals in oestrus were mated with a Dorset ram. Mating activity was monitored with a raddled ram and pregnancy confirmed at about 60 days post mating by transabdominal ultrasound. Mated ewes (which were assumed and then confirmed pregnant) were injected i.m. twice weekly with 100 mg TP or DHT in vegetable oil from 30–90 days of pregnancy (term 147 days). The dose and mode of testosterone and DHT administration were chosen to reflect the large body of data available relative to postnatal reproductive disruptions (Masek *et al.*, 1999; Robinson, 2001; West *et al.*, 2001; Manikkam *et al.*, 2006; Steckler *et al.*, 2007b). Prenatal TP treatment produces circulating concentrations of testosterone in female foetuses in the range observed in male foetuses (Wood et al., 1991). Ewes giving birth to control offspring for these studies received no treatment as it has been established that the response of their offspring does not differ from that of offspring to mothers injected with vehicle (Steckler et al., 2007). Studies were performed on five groups of ewes, one group per year, which were born to mothers who had been exposed during their pregnancy to TP, DHT or to no hormone. Year groups of sheep will be referred to in this thesis as year group 1 for year 1, year group 2 for year 2

etc. Year groups 1-4 contained two treatments, either control ewes or ewes treated with TP *in utero*, while year group 5 ewes contained an additional *in utero* DHT treatment group.

The groups of sheep used in the study are illustrated in Table 2.1

Year Group	Age at Slaughter	Date of Slaughter	No. of control ewes	No. of TP ewes	No. of DHT ewes
1	22 months	February 2005	9	7	0
2	12 months	April 2006	7	6	0
3	10 months	February 2007	7	10	0
4	10 months	March 2008	7	6	0
5	10 months	March 2009	14	15	8

Table 2.1. Details of the groups of sheep used in the studies. Sheep spanning 5 years (2005-2009 inclusive) were used.

2.3 Blood Sampling

Blood samples were collected from control and androgenised ewe lambs by jugular venipuncture twice weekly from approximately 25 weeks of age which was in October/November (puberty ~30 weeks – Quirke *et al.*, 1985) until the end of February in all five groups of sheep. Dorset ewes normally enter the anoestrous season in January at this latitude (55°55'N). All blood samples were collected by Dr Peter Hastie, Dr Jane Robinsion and Prof Neil Evans. Blood was collected into tubes containing heparin (1 drop of 5000IU/ml per tube) as anticoagulant. Immediately after collection plasma was separated via centrifugation (usually 3000 rpm for 15 minutes at 4°C) and stored at -20°C until required for assay. The occurrence of ovulatory cycles was determined from measurements of progesterone in plasma, and peripheral oestradiol concentrations were investigated as short-term experiments indicated that concentrations are elevated in the prenatally TP-treated Suffolk ewe (Veiga-Lopez *et al.*, 2008).

2.4 Collection of Ovaries and Isolation of Granulosa cells

Ewes were euthanased by lethal barbiturate overdose (Somulose, 1ml/10kg BW, Dechra Veterinary Products, Shrewbury, UK). The euthanasia procedure was performed by either Dr Peter Hastie or Dr Jane Robinson. Ovaries were recovered from each ewe, cleaned of blood and surrounding excess tissue, weighed, and ovarian maps drawn detailing the location and number of large antral follicles in addition to noting the presence or absence of any *corpora lutea.* All follicles greater than 3.5 mm in control and DHT ewes and

greater than 5 mm in TP ewes were excised free from stroma and measured using callipers. Follicles of this diameter were chosen for excision as in the normal sheep, follicles >3.5 mm in diameter are gonadotrophin dependent and likely to contain the dominant follicle with highest oestradiol synthesizing capacity (Dufour *et al.*, 1979; Miller *et al.*, 1979; McNeilly, 1984; McNeilly *et al.*, 1992). As ovaries from ewes androgenised *in utero* with TP (but not with DHT) contain many follicles much larger than 3.5 mm (Smith *et al.*, 2008), an increased diameter threshold was set at 5 mm in order to recover the putative dominant follicle. In most ewes (control - 29/35, TP – 28/37 and DHT – 6/8 ewes), two follicles per ewe were excised using such diameter thresholds, while only one (in 6/35 controls, 7/37 TP-treated, and 2/8 DHT-treated ewes) or three follicles (in 2/37 TP-treated ewes) were recovered from the remaining experimental ewes. Follicular fluid was aspirated gently from all the dissected follicles using a 25 gauge needle attached to a 1 ml syringe to minimise aspiration of live granulosa cells. Follicular fluid was immediately frozen in liquid nitrogen and then stored at –80°C until used for steroid hormone analysis. Granulosa cells were isolated and collected by hemisecting follicles and using a plastic inoculation loop to gently scrape the cells from the follicle wall (Glister *et al.*, 2001). Cells were washed in Phosphate Buffered Saline (PBS) (or culture media; for details of culture media see section 2.8), and collected by centrifugation at 1000 rpm for 5 minutes. After centrifugation, the granulosa cell pellet was either re-suspended in 500 µl TRIzol (Invitrogen, Paisley, UK), snap frozen and stored at -80°C until RNA extraction, or re-suspended in PBS or media for subsequent cell culture. Time from euthanasia of the ewe to pelleting of the granulosa cells was less than 1 hour. Please note, that for year groups 1 and 2 ovaries were collected and follicles excised by Dr Monika Mihm and Dr Paul Baker.

2.5 Follicle Classification

Based on follicular fluid oestradiol concentration of the two largest follicles recovered from each ewe (or the three largest follicles recovered in 2/37 TP-treated ewes), follicles were classified as the dominant follicle (DF - with higher intrafollicular oestradiol) or the subordinate follicle (SF - lower intrafollicular oestradiol) (Evans & Martin, 2000; Austin *et al.*, 2001; Ryan *et al.*, 2007). DF have the potential to continue to ovulation, while SF regress. However, in the case of a sheep breed with an ovulation rate of 1.8 (Hall *et al.*, 1986), the two largest follicles in control sheep may also represent two follicles with the ability to ovulate following luteolysis, despite some differences in oestradiol synthesizing capacity. Thus the SF classification in control ewes may include some co-dominant follicles (Fortune *et al.*, 2004). In addition, largest follicles with lower intrafollicular oestradiol than the second largest follicle may also represent regressing dominant follicles.

Again this possibility can not be excluded from our classification of control SF, although the bovine literature shows that regressing DF have very high intrafollicular progesterone concentrations (Sunderland *et al.*, 1994) which was not the case here (see results Chapter 3). Follicles classified as dominant based both on highest oestradiol and largest follicle diameter out of the follicles recovered occurred in 59% (17/29), 57% (16/28) and 50% (3/6) of control, TP- and DHT-treated ewes, respectively. When only one follicle was recovered per ewe, then that follicle was classified as DF independent of intrafollicular oestradiol concentrations, as there was no evidence that another follicle was present that was larger (with higher follicular fluid oestradiol) and therefore potentially a dominant follicle.

2.6 RIA Procedures for determination of steroid concentrations

Radioimmunoassay (RIA) is a competitive technique developed in 1960 for measuring very small concentrations of a substance (Yalow & Berson, 1960). The basic theory of an RIA is that the hormone, e.g. oestradiol, in the samples/standards will compete with a known amount of iodinated (radioactive) oestradiol to bind to a limiting amount of a specific oestradiol antibody. Thus, the percentage of iodinated oestradiol bound to the antibody will decrease as the concentration of unlabelled (non-radioactive) oestradiol within the sample/standard increases. The amount of unlabelled oestradiol bound in unknown samples is then indirectly quantified by comparison to the displacement of iodinated oestradiol by known standards. In all RIA's the limit of assay sensitivity was assigned to those samples in which the concentration of steroid was below the sensitivity of the assay. All reagents and chemicals were obtained from Invitrogen Ltd. (Paisley, UK) unless otherwise stated.

Dilutions

Concentrations of the steroid hormones oestradiol and progesterone in plasma, the follicular fluid of the largest antral follicles and conditioned culture media were determined using radioimmunoassay (RIA). Testosterone concentration was determined only in follicular fluid. All follicular fluid samples were diluted in MAIA buffer (0.01 M PBS, 0.1% gelatin, and 0.1% sodium azide, pH 7.0). Follicular fluid samples were diluted 1:100-1:1000 for oestradiol, 1:10 – 1:100 for progesterone and 1:100 – 1:1000 for testosterone concentration determination. Follicular fluid samples for oestradiol, progesterone and testosterone assays required no prior extraction. A volume of 10-200 µl and 100 µl of plasma was used to determine oestradiol and progesterone concentrations, respectively in the peripheral circulation. Plasma samples for progesterone assay required no prior

extraction, while 200 µl of plasma was extracted in duplicate to determine peripheral oestradiol concentrations. A volume of 10-200 µl of conditioned media was used to determine oestradiol and progesterone concentrations in conditioned media. Conditioned media for oestradiol and progesterone assays required no prior extraction.

Oestradiol RIA - Assay Reagents and Procedure

Oestradiol concentrations were determined using a commercial RIA kit (MAIA Oestradiol Kit, Bio-Stat Limited, Stockport, UK. Cat no. 370001). The primary antibody (rabbit antiserum) was diluted to 1:10 with MAIA assay buffer (0.01 M PBS, 0.1% gelatin, and 0.1% sodium azide, and adjusted to pH 7.2) prior to use. The reference preparation was diluted to give ten standard curve points ranging from 0.195-100 pg/ml (0.039-20 pg/tube). The secondary antibody used was anti-rabbit antiserum, raised in goat, and was supplied ready diluted. The secondary antibody is coupled to magnetic particles to facilitate recovery of the oestradiol bound to the primary antibody. Samples pooled from each year group were used in each assay as quality controls.

Plasma oestradiol concentrations were measured in year groups 1-4 using a validated method (Evans et., al 1994). Briefly, duplicate 200 µl of plasma samples for each date (see Chapter 3) and steroid standards were extracted in 16x100 mm borosilicate glass tubes (Fisher Scientific UK Ltd, Loughborough, UK) using 2 ml diethyl ether (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK). Samples were vortexed for 15 minutes then frozen for 5-10 seconds in a methanol dry ice mixture. The liquid fraction was poured off, dried down to remove remaining ether by evaporation, re-suspended in 300 µl of MAIA buffer and briefly vortexed for 1 minute. The serum oestradiol concentrations were then determined using the assay procedure and reagents as detailed below. For determination of oestradiol in follicular fluid and conditioned media duplicate sets of standards and samples were added to MAIA buffer in a small volume for a final assay volume of 300 µl.

Non-specific binding tubes (in duplicate) containing 300 µl of assay buffer and 50 µl of normal rabbit serum (1:200) were added. All other tubes received 50 µl of the diluted primary antibody (1:10) except those for total counts and non-specific binding, and were vortexed and incubated for 1 hour at room temperature. Next, 50 µl of ^{125}I-oestradiol (range 9000 -12500 cpm) were added to all tubes, which were then vortexed and incubated for two and a half hours at room temperature. The total counts tubes were capped and set aside at this point. Finally, 250 µl of the magnetically linked secondary antibody were added to all tubes, which were vortexed and incubated at room temperature for 20 minutes.

Following incubation, all tubes were placed in magnetic racks for 20 minutes during which time the magnetic particles linked to the secondary antibody (bound to the first antibody and oestradiol) are pulled to the bottom of each tube. Next, while the tubes are in the magnetic racks the supernatant was poured off by inverting the racks. Following this, the tubes were blotted on soft absorbent paper and 1 ml dH_2O was added to all tubes to wash away any unbound primary antibody and left for 10 minutes. Next, the magnetic racks holding the tubes were inverted again to pour off the supernatant, and the tubes were blotted well on soft absorbent paper. Following the second round of blotting, the tubes were counted using a gamma counter for 90 seconds and average cpm were calculated (Cobra™ II Auto-Gamma®, Packard Bioscience UK).

Progesterone RIA - Assay Reagents and Procedure

Progesterone concentrations were measured using a commercial Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA). Duplicate sets of standards were made up to 100 µl with assay buffer, while 100 µl of the unknown samples were added to each tube. Thus either 100 µl of undiluted plasma, or follicular fluid or conditioned media samples were diluted in assay (MAIA) buffer to give a total volume of 100 µl were then added to the assay. The reference preparation was diluted to give eleven standard curve points ranging from 0.039 – 40 ng/ml. The tubes used in this assay have the primary antibody attached to them. Non-specific binding tubes (in duplicate) contained 100 µl assay buffer. In the case of total count and non-specific binding tubes, plastic tubes without the primary antibody attached to them were used. A volume of 750 µl of ^{125}I-Progesterone (~ca. 38000 cpm) was added to all tubes, which were then vortexed and incubated for 3 hours at room temperature. Next, tubes were placed in magnetic racks except those for total counts, the supernatant was poured off by inverting the rack, and tube openings were blotted several times on soft absorbent paper to remove any unbound ^{125}I-Progesterone. Following blotting, the tubes were counted for 60 seconds using a gamma counter (Cobra™ II Auto-Gamma®, Packard Bioscience UK).

Testosterone RIA - Assay Reagents and Procedure

Follicular fluid concentrations of testosterone were measured without prior extraction using an established assay (Sheffield & O'Shaughnessy, 1989). Duplicate sets of standards and appropriate follicular fluid dilutions were added to MAIA buffer in a small volume for a final assay volume of 100 µl. The reference preparation was diluted to give eleven standard curve points ranging from 0.01 – 10 ng/ml. Tritiated testosterone was diluted from stock in MAIA buffer to give approximately 3000 cpm. The primary antibody (rabbit

antiserum) (GE Healthcare, Amersham Place, Buckinghamshire, UK) was diluted to 1:900 in MAIA buffer, and 100 µl of primary antibody were added to all tubes, except total count and non-specific binding tubes. Non-specific binding tubes (in duplicate) contained 200 µl assay buffer. Next, 50µl of ^{3}H-testosterone were added to all tubes, which were then vortexed and incubated for 2 hours at room temperature. Following incubation, 400 µl of dextran charcoal buffer (0.2 g/100 ml of MAIA buffer) was added to all tubes (except total count tubes), which were vortexed and left for 10 minutes. All tubes except the total count tubes were then centrifuged at 3,000 rpm for 10 minutes at 4°C. The dextran charcoal buffer enables separation of bound and free labelled hormone and thus in this assay binds the free radioactive hormone. The principle of the method is that free labelled hormone will bind to the charcoal particles and by suspending the charcoal in appropriate media the antibody-bound labelled hormone can be excluded. Removal of the supernatant after centrifugation of the charcoal suspension allows the separated bound radioactive hormone to be counted (Jacobs, 1969). The supernatant was decanted into vials containing 4 mls of scintillation fluid. Radioactive isotopes react with the scintillation fluid to produce light and these light emissions are detected by a scintillation analyzer. The vials were capped, shaken and then counted for 60 seconds using a liquid scintillation analyzer (1600TR liquid scintillation analyzer, Packard Bioscience, UK).

2.7 Molecular Biology

Where relevant, RNase-precautions were adopted. Hence gloves were worn at all times and changed frequently. All pipette tips used were certified RNase, DNase and protease free. Ninety-six well plates, eppendorf tubes and molecular grade water were all exposed to UV light prior to use. Molecular biology grade water was used in all enzymatic reactions.

RNA Extraction

The extraction of RNA from granulosa cells was performed using the TRIzol reagent (Invitrogen Ltd, Paisley, UK) according to the manufacturer's protocol. TRIzol reagent is a mixture of guanidine thiocyanate and phenol in a mono-phase solution which dissolves DNA, RNA and protein following the lysis of cells or homogenization of tissue. Guanidine thiocyanate is a chaotropic agent, disrupting the hydrogen bonds between biological molecules. Thus, its addition disrupts the cell membrane, enabling cell lysis, denatures proteins including RNases, and separates rRNA from ribosomes. Depending on the year group, granulosa cell preparations were re-suspended in 1ml of TRIzol reagent prior to freezing or granulosa cell pellets were removed from the -80°C freezer, TRIzol reagent was added and cells were then pipetted several times to homogenise the lysates. Cell samples

required minimal homogenization since the TRIzol reagent disassociated most of the tissue. To ensure complete dissociation of nucleoprotein complexes samples were allowed to stand for 5 minutes at room temperature. Chloroform (0.2 ml of chloroform per 1 ml of TRIzol reagent) was added to each sample, which was then shaken vigorously for 15 seconds and allowed to stand at room temperature for a further 5 minutes. Centrifugation at 12,000 rpm for 15 minutes at 4°C separated the TRIzol reagent cell mixture into 3 phases. The lower phase contains protein, the interphase contains DNA and the colourless aqueous upper phase contains RNA. The upper phase was transferred into a new tube for RNA precipitation and the rest discarded. Isopropanol (0.5ml of isopropanol per 1 ml of TRIzol reagent) was added to each sample, mixed, and then incubated at room temperature for 10 minutes before being centrifuged at 12,000 rpm for 10 minutes at 4°C. Centrifugation results in the RNA precipitate forming a pellet at the bottom of the tube. The supernatant was then removed taking care not to remove any of the pellets. Ice cold 70% ethanol (1 ml of 70% ethanol per 1 ml of TRIzol reagent) was added and the sample vortexed briefly to wash the RNA pellet. The sample was then centrifuged at 12,000 rpm for 10 minutes at 4°C. Supernatant was poured off and the RNA was dried briefly for 5-10 minutes at room temperature and re-suspended in 20 µl of RNase-free water. Extracted total RNA samples were stored at -80°C after quantification (see below).

RNA extraction before and after granulosa cell culture

Due to the fact that a relatively low number of granulosa cells were available for seeding from individual experimental follicles, and few live cells were recovered after 6 days of culture, a specialized commercial RNA extraction kit (RNAqueous®-Micro Kit, Ambion, Warrington, UK), optimized for the purification of total RNA from micro sized samples such as 10–500,000 cultured cells was used to extract RNA from all granulosa cells recovered before and after culture in Year group 5. The extraction was performed exactly according to manufacturer's protocol. Briefly, uncultured and cultured granulosa cells (pelleted) were re-suspended and mixed in 100 µl lysis solution containing guanidinium thiocyanate, a strong chaotropic agent disrupting cell membranes and rapidly inactivating ribonucleases. The lysate was then mixed with ethanol and applied to a silica-based filter that selectively binds RNA. The lysate/ethanol mix was centrifuged at 14,000 rpm (maximum speed) for 10 seconds. The RNA is now bound to the silica-based filter. Proteins, DNA, and other contaminants were removed in three rapid ethanol washing steps. The first washing step entailed the addition of 180 µl of ethanol wash solution 1 to the silica-based filter, followed by centrifugation for 10 seconds at 14,000 rpm. The second and third washing steps required the addition of 180 µl of ethanol wash solution 2 to the

silica-based filter followed by centrifugation for 10 seconds at 14,000 rpm. The bound RNA was eluted in concentrated form in a volume of 20 µl of elution solution and quantified (see below).

DNase I Treatment

Isolated total RNA was DNase I treated (Ambion, Warrington, UK) as per manufacturer's protocol to remove DNA contamination from RNA preparations. DNase I is an enzyme that non-specifically cleaves DNA to release 5'-phosphorylated di-, tri-, and oligonucleotide products thus degrading contaminating DNA from the RNA preparation. A volume of 5.3 µl of analytical grade H_2O, 1.2 µl of DNase buffer (x10) and 0.5 µl of DNase I were added to a 0.5 ml eppendorf tube. To this mix, 5 µl of isolated RNA (corresponding generally to 1 µg of RNA (range 0.7-1.2 µg) was added and incubated at 37°C for 40 minutes. To stop the reaction 4 µl of reaction stop mix was added, the preparation vortexed and incubated for 1 minute. The reaction stop mix removes the DNAse I and divalent cations (e.g. Mg^{2+}) from the sample thus stopping the enzymatic activity of DNAses. The preparation was centrifuged at 14,000 rpm for 1 minute, the reaction stop mixture containing the DNase I, fragments of the genomic DNA and cations forming a pellet at the bottom of the tube, facilitating easy removal of the supernatant containing the DNase I-treated total RNA into a new eppendorf tube. All DNase I treated samples were stored at -80°C prior to RNA quantification by spectrophotometry (see below) and reverse transcription.

Reverse Transcription PCR

Reverse transcription is a natural process that creates single stranded complementary DNA (cDNA) from an RNA template using the enzyme reverse transcriptase (Bustin, 2000). Complementary DNA was synthesized from DNase I treated total RNA isolated from granulosa cells of individual follicles using the SuperScript III First-Strand Synthesis System (Invitrogen Ltd, Paisley, UK), according to the standard protocol provided. The 50 µl reaction mixture comprised 300 ng of total RNA, 690 pmols of random hexamer primers, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 5 x first strand buffer, 0.1 M dithiothreitol, 30 U RNase inhibitor and 150 U SuperScript III reverse transcriptase (all reagents from Invitrogen, Paisley, UK). Briefly, 300 ng of total RNA was primed with random hexamers. The priming mixture was incubated at 65°C for 5 minutes to denature RNA secondary structure, and then immediately chilled on ice enabling random hexamers to bind to the complementary sequence. The reverse transcription reaction was carried out at 50°C for 50 minutes to allow extension of the synthesis strand by the reverse

transcriptase enzyme, and afterwards the enzyme was inactivated by incubating at 70°C for 10 minutes. To act as a negative control, the SuperScript III reverse transcriptase enzyme was substituted with sterile nuclease-free water. A negative control sample was included only for a subset of samples from every year group, because in some granulosa cell preparations, due to the very small number of cells isolated, the low amounts of RNA extracted were not sufficient for a negative reverse transcription control.

Quantification of Nucleic Acids

The quantity and quality of DNase I treated total RNA and reverse transcribed cDNA were assessed using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Ltd., Delaware, USA) to measure optical density (OD) at wavelengths of 230 nm, 260 nm and 280 nm. Nucleic acids absorb light at 260 nm, while proteins absorb light at 280 nm. Absorption at 230 nm corresponds to organic compounds such as thiocyanates, (e.g. the guanidinium thiocyanate contained within TRIzol reagent that was used to isolate the RNA, phenol used in the extraction and ethanol used to precipitate the cDNA). Concentration of RNA was quantified by measuring absorbance at 260 nm using a spectrophotometer and calculated as follows: RNA concentration (μg/μl) = (OD 260) x (dilution factor) x (40 μg RNA/ml)/ (1 OD260 unit). Concentration of cDNA was calculated as follows: DNA concentration (μg/ml) = (OD 260) x (dilution factor) x (50 μg DNA/ml) / (1 OD260 unit). High purity of a sample was indicated by an OD260 nm/ OD280 nm ratio reading of approximately 1.8 for DNA and approximately 2.0 for RNA. A secondary measurement of nucleic acid purity is provided by the OD260 nm/ OD230 nm ratio; a value lower than 1.8-2.2 suggests the presence of organic compound contaminants.

Semi-quantitative Real-Time PCR

Overview of the technique

The essential components in a PCR amplification reaction include the target cDNA, Taq polymerase, two locus specific oligonucleotide primers, deoxynucleotide triphosphates, reaction buffer and Mg^{2+}. The first step in the reaction is denaturation where a high temperature is used to melt the double-stranded RNA-cDNA duplex into the single-stranded cDNA (in later cycles double-stranded amplified DNA is melted). Primer annealing follows at a lower temperature, which is optimised (as high as possible) to prevent binding of primers to non-specific binding sites (non-complementary nucleotide sequences) on the target cDNA. The final step in the process is extension, where Taq polymerase extends the double strand from the primer by adding complementary nucleotides in a 5'-3' direction. Each thermal cycle doubles the amount of target sequence

in the reaction. The cycle is repeated multiple times (usually 35-40 times), amplifying the copies of the target DNA exponentially (2^{35-40}).

Real-time PCR is a method which allows amplification of DNA by PCR to be monitored while the amplification is occurring (VanGuilder *et al.*, 2008; Logan *et al.*, 2009). The major advantage of this is that it allows relative or absolute (based on a standard curve) quantification, i.e. determination of the amount of template cDNA in the sample before amplification by PCR (VanGuilder *et al.*, 2008; Logan *et al.*, 2009). The technique is specific and extremely sensitive, capable of detecting very low levels of mRNA gene expression.

In this study, relative quantification was used to determine changes in gene expression. Relative quantification determines steady-state mRNA levels of a target gene across multiple samples and expresses it relative to the levels of mRNA for one or more reference gene(s) (housekeeping genes). Thus the technique is suitable for investigating physiological changes in gene expression levels. Therefore, relative quantification does not require pure standards with known concentrations, but the sequence must be known for both reference and target transcript species (Schmittgen & Livak, 2008).

Relative quantification in the study presented was achieved using the Brilliant™ SYBR Green I Master Mix (Stratagene, La Jolla, USA) used in conjunction using an Mx3000P real-time PCR system (Agilent Technologies UK Ltd, Stockport, UK). SYBR Green I is a fluorescent dye, which binds non-specifically to double-stranded DNA (dsDNA). It exhibits little fluorescence when it is free in solution, but its fluorescence increases up to 1,000-fold when it binds dsDNA. Following each cycle of amplification in the PCR reaction, the fluorescence of the samples is determined, providing an indirect measure of the number of amplified (double stranded) sequences. Subsequently, the cycle threshold value (C_t) is determined, which is defined as the number of cycles required to reach a minimum (predetermined) fluorescent threshold. The threshold is ideally set at the beginning of the exponential phase of amplification to ensure that efficiency of each reaction is maximal and not influenced by compound depletion in the reaction. For relative quantification of mRNA concentrations the comparative C_T method was used, wherein the mRNA expression of each gene of interest was quantified relative to the mRNA expression of the endogenous reference gene ACTB (User Bulletin no. 2, PE Biosystems, UK). Validation experiments confirmed that the amplification efficiencies of the genes of interest and *ACTB* were comparable, the slope of the difference between C_T values for each standard concentration from ACTB and the gene of interest were <0.1 (that is the slopes

were parallel) and, thus, gradients of standard curves of genes of interest were within 5% of the *ACTB* slope (Figure 2.1). Parallelism of standard curves is important when using the relative quantification analysis for mRNA expression (i.e. expression levels are related to a reference gene), because if there is a disparity in the amplification efficiencies between the reference gene and the gene of interest, the delta C_T values (estimating expression relative to the reference) will differ due to differences in amounts of template added, rather than only due to sample differences in mRNA expression (Schmittgen & Livak, 2008).

Gene	Amplification efficiency of standard curves	Accession Number	Amplicon Size (base pairs)	Melting Temperature of Amplicon (Tm)
ACTB	97%	U39357	73	79°C
LHR	99%	L36329	109	76°C
FSHR exon 4/5	96%	L07302	94	77°C
FSHR exon 9/10	98%	L07302	110	80°C
CYP19A1	95%	AJ012153	96	75°C
HSD3B1	100%	X17614	78	78°C
INHA	97%	L28815	189	87°C
INHBA	96%	NM_001009558	72	80°C
FST	98%	M63123	122	85°C
MIF	95%	XM_001033608	87	83°C
CCND2	100%	NM_001076372	101	82°C
MCL-1	98%	NM_001099206	51	74°C
CAPS3	96%	NM_001077840	87	75°C
BAX	99%	NM_173874	182	84°C

Table 2.2 PCR amplification efficiencies for the genes of interest used, as calculated by determining the cycle threshold number for 5 cDNA concentrations (625, 125, 25, 5 and 1 ng) added as a template.

Real-Time Quantitative PCR reaction

The Brilliant™ SYBR Green Master Mix (Stratagene, La Jolla, USA) in conjunction with the ThermoCycler System (Stratagene, La Jolla, USA) was used to measure mRNA expression. Real-time primer sets were designed from species-specific sequences of genes using Primer Express Software v2.0.0 (Applied Biosystems), synthesised by Eurofins MWG Operon (Ebersberg, Germany), and used at a concentration of 1000 nM. Primers were designed across intron boundaries to avoid amplification of genomic DNA (should any remain in samples after DNAse treatment). Reactions were carried out in duplicate using 96-well PCR plates in a final reaction volume of 10 µl made up of 5 µl Brilliant™ SYBR Green I Master Mix (Stratagene, La Jolla, USA), 2 µl primer mix and 3 µl cDNA (50 ng year groups 2 and 3, 400 ng year groups 4 and 5) template or negative control. The plates were sealed after addition of the SYBR, template and primer mix and then centrifuged at 1000 rpm for 1 minute. To facilitate optimisation and troubleshooting, both negative control and a positive quality control sample were included in all reactions. To act

as a negative control, cDNA template was substituted with sterile nuclease-free water. As a positive control, a sample of pooled cDNA was used and run for each gene of interest on each plate. The standard thermal profile set-up within the thermal cycler was used for all of the genes investigated and is shown in Figure 2.3. Note that an initial phase (segment 1) of 95°C for 10 minutes is used to activate the hot start DNA polymerase, thus allowing reactions to be set up at room temperature. Heating at this temperature denatures proteins around the active site of the DNA polymerase, thus allowing access to template and to free dNTPs. Primer annealing temperature was determined empirically through numerous trials using different annealing temperatures (data not shown) but were based on the melting temperatures (T_m's) supplied by the primer express software (58 to 60°C), with annealing temperatures generally 3-5°C lower than the T_m. Excitation and emission maxima of the Brilliant™ SYBR Green Master Mix are at 497 nm and 520 nm, respectively; therefore, fluorescence emission was measured after each primer extension step at 520 nm. Reactions with primer pairs to amplify the normalising reference gene and the gene of interest were always run on the same plate.

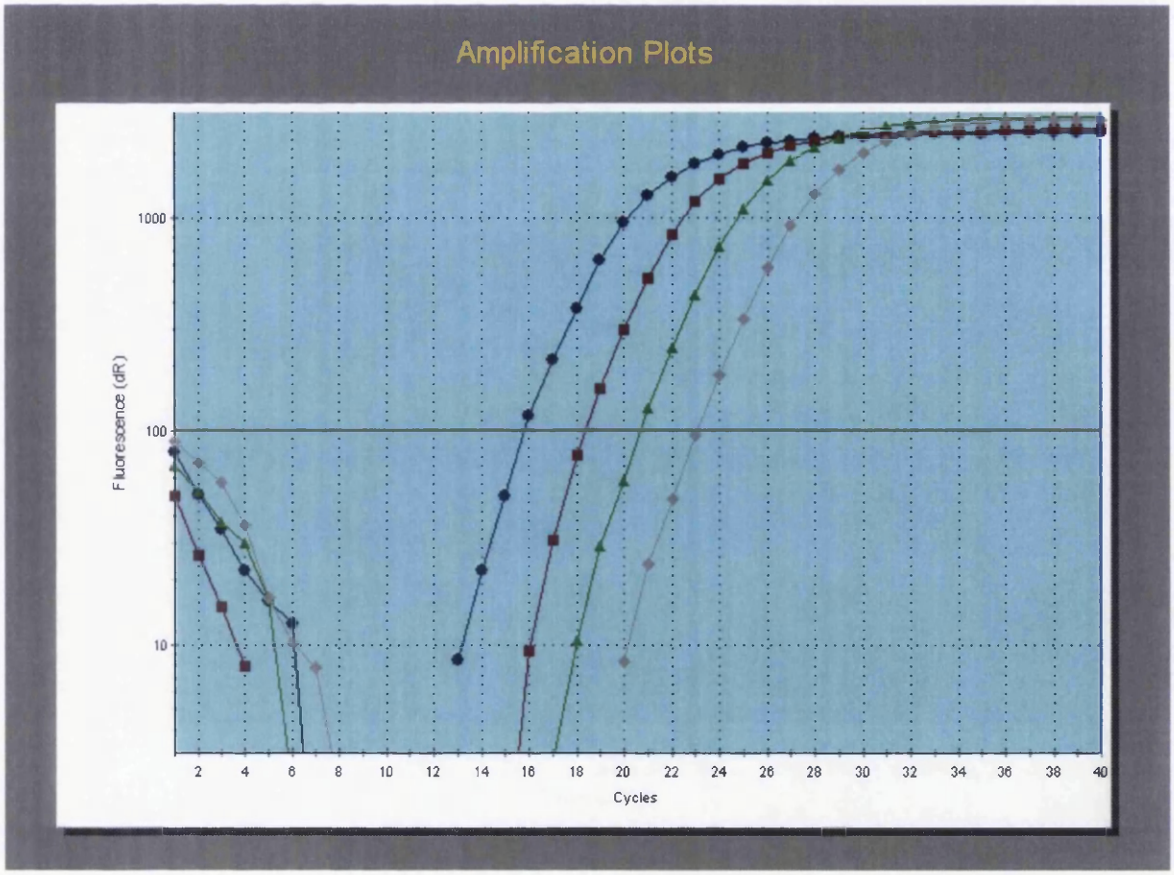


Figure 2.1 Fluorescence of standards during PCR amplification of *CYP19A1* transcripts. From left to right the cDNA template standard concentrations are 625 ng (blue), 125 ng (red), 25 ng (green) and 1 ng (grey) per well. The threshold bar (brown horizontal bar) was used to measure the cycle threshold number (C_T) for each standard in order to create a standard curve and determine the amplification efficiency for each gene of interest.

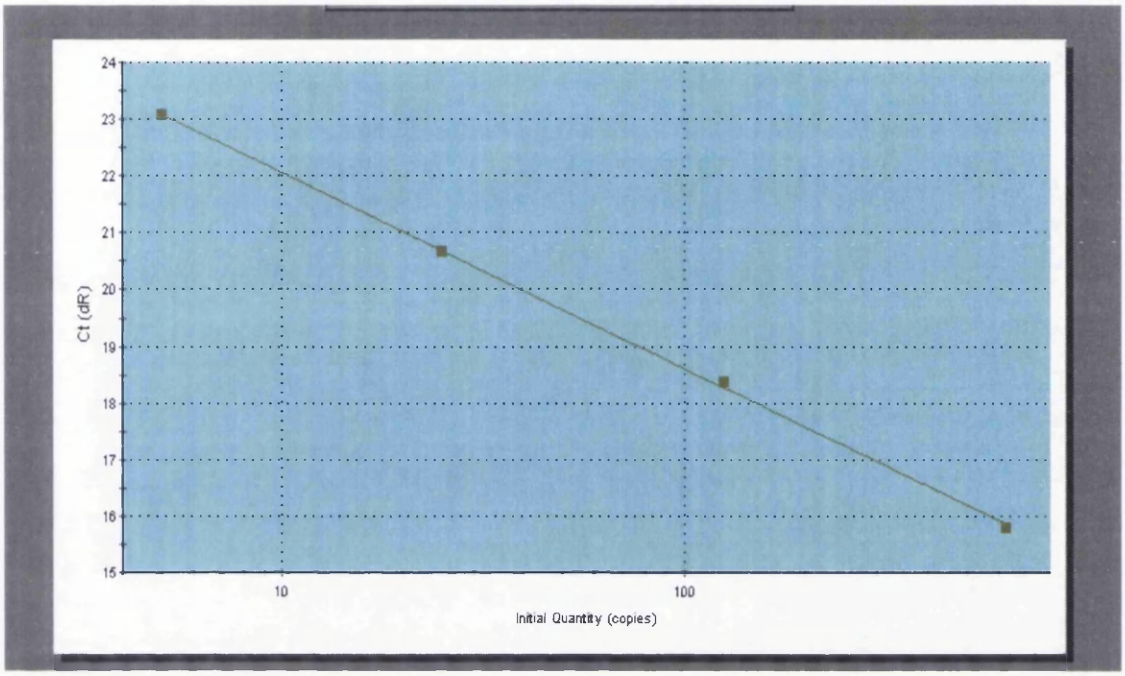


Figure 2.2 Standard curve of initial quantity and cycle number at which fluorescence levels crossed the threshold fluorescent intensity during the PCR amplification of *CYP19A1*. The sample threshold cycle number at each cDNA template standard concentration was used to determine the efficiency of the PCR reaction, ensuring amplification efficiences were 95-100% for each gene of interest and similar to that of the *ACTB* PCR efficiency.

Melting Curve Analysis

During the melting step the temperature was increased by small increments and the fluorescent emission was measured continuously. SYBR Green I binds only to the minor groove of double stranded DNA, so dissociates as the PCR product begins to denature, resulting in a dramatic decrease in fluorescence. A melting curve is produced, which represents the rate of change in fluorescence as temperature increases, therefore peaks represent temperatures at which there is a rapid melting of specific PCR products (Figure 2.4). Peaks at a lower T_m than that of the specific PCR product expected indicate the melting of primer-dimers which can form during PCR, while several peaks with a range of T_ms suggest the formation of non-specific products. Therefore, the presence of a single peak for all products indicates that the product is pure and specific if the T_m is in accordance to the one predicted. The intensity of the fluorescent signal differs between samples as a result of differing quantities of the DNA at the end of the reaction between each sample. Frequently, very small peaks at very high temperatures were observed in all reactions, and considered to be artefacts.

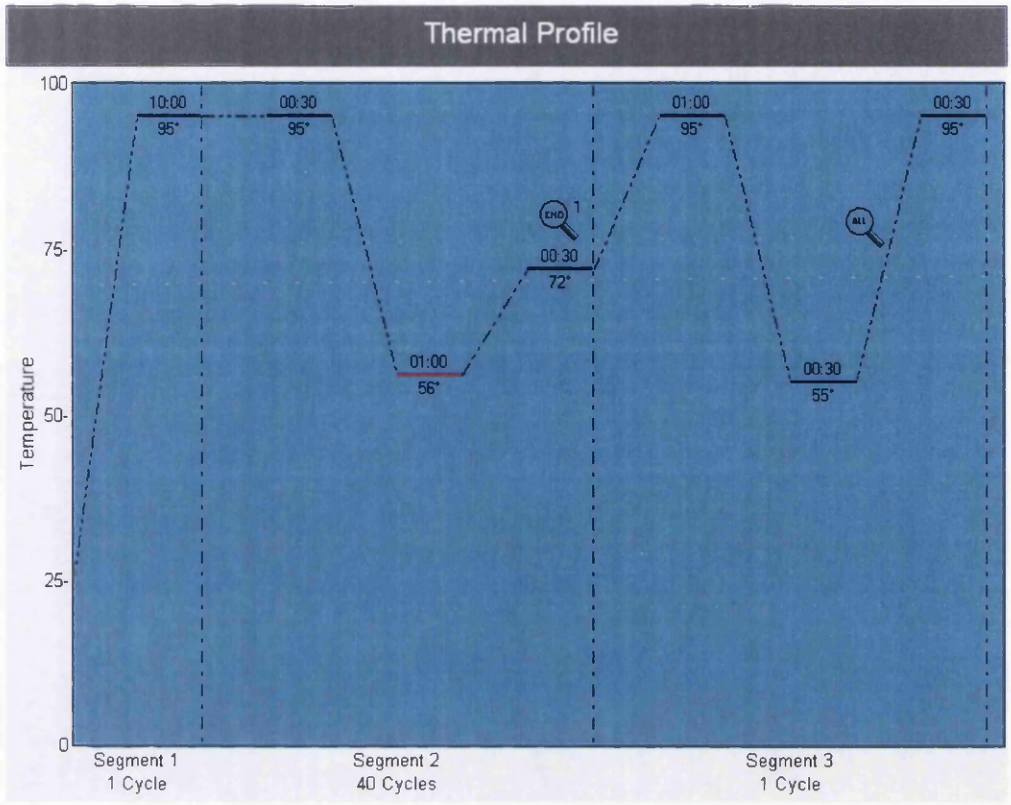


Figure 2.3 The standard thermal profile used for all of the genes investigated using real-time PCR. Initially, the SureStart *Taq* DNA Polymerase in the Brilliant™ SYBR Green I Master Mix is activated by heating at 95°C for 10 minutes (segment 1). This denatures the proteins surrounding the active site of the DNA polymerase allowing free dNTP access. Following activation of the DNA Polymerase, target amplicon amplification is achieved by 40 sequential cycles of denaturing (95°C for 30 seconds), primer annealing (56°C for 1 minute) and extension (72°C for 30 seconds). Fluorescence levels are measured after the extension step (END) (segment 2). Finally, the dissociation (melting) curve of the generated products is determined by denaturing (95°C for 1 minute), annealing of all double-stranded products (55°C for 30 seconds) and then gradual denaturation of duplexes up to a temperature of 95°C. During the gradual increase in temperature, fluorescence levels are measured continuously (ALL) to determine the melting temperature of the specific PCR product which is compared with that predicted from the primer express programme.

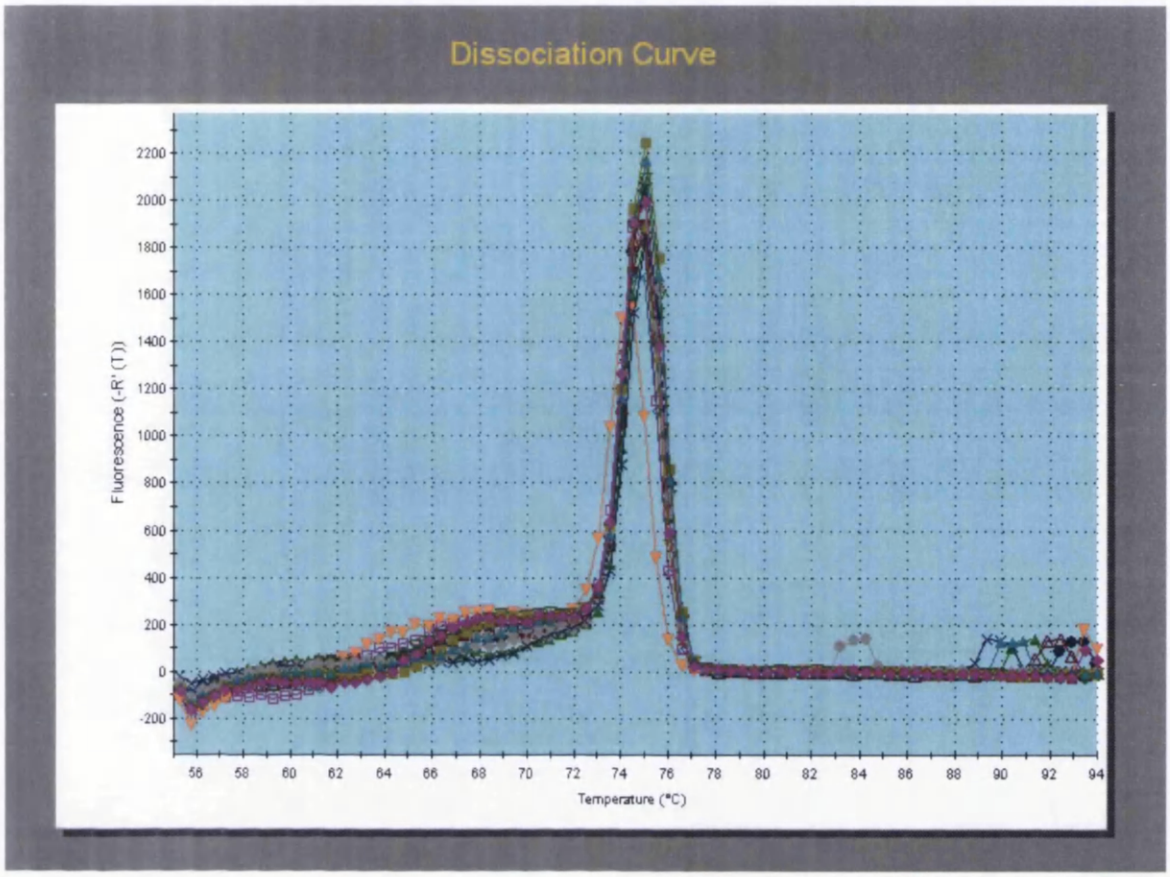


Figure 2.4 Dissociation (melting) curve analysis following PCR amplification of the *CASP3* gene. The peak represents the temperature at which the greatest rate of change in fluorescence (high to low) occurs as the temperature increases, resulting in double stranded cDNA melting apart to become single stranded. In this example, the melting temperature of the *CASP3* amplicon was 75°C which was identical to the predicted T_m.

2.8 GRANULOSA CELL CULTURE

Sheep ovaries were collected from the abattoir for control setups, and from experimental control and prenatally androgenised ewes and placed in transport media (M199 medium, supplemented with 1% (vol/vol) antibiotic-antimycotic solution and 3 mM l-glutamine (all reagents from Sigma Ltd, Poole, UK). Ovaries were trimmed and placed in 70% ethanol for 30 seconds, then rinsed and maintained in transport media until dissection. Follicles were excised, follicular fluid aspirated, and granulosa cells recovered as described in section 2.4. Granulosa cells scraped in 500 µl of zero supplemented media (McCoy's 5A modified medium supplemented with 1% (vol/vol) antibiotic-antimycotic solution, 10 ng/ml bovine insulin, 10 ng/ml Human long R^3 IGF-1, 3 mM l-glutamine, 20 mM HEPES, 5 µg/ml apotransferrin, 5 ng/ml sodium selenite, and 0.1% BSA; all purchased from Sigma Ltd, Poole, UK) were pelleted by centrifugation at 1,200 rpm for 5 minutes followed by removal of the supernatant. All subsequent work was carried out in a laminar flow hood under sterile conditions. Red blood cells were lysed by re-suspending the pellet in 200 µl of dH_2O followed by the addition of 800 µl of 0.1M PBS. Granulosa cells were washed twice by re-suspending the pellet in 500 µl of zero supplemented media,

centrifuging for 5 minutes at 1,200 rpm followed by removal of the supernatant. Following the second wash, granulosa cells were re-suspended in 410 µl of zero supplemented medium. Ten microlitres of the granulosa cell suspension was used to determine the number of live cells by Trypan blue exclusion as described below. Following isolation and washing granulosa cells were incubated in hormone supplemented medium at 37°C in a humidified 5% CO_2 atmosphere for 6 days. After 48 and 96 hours of culture, approximately 200 µl conditioned media was removed from each well very slowly to minimise disruption to the granulosa cells, stored at minus 20°C until RIA and replaced with 200 µl of the appropriate fresh media. Fresh media was placed in the incubator for at least 2 hours prior to replacement of conditioned media to allow equilibration. At 144 hrs of culture, 200 µl conditioned media was removed very slowly, stored at -20°C and granulosa cell viability determined by Tyrpan blue exclusion (see below).

2.9 Determination of numbers of live cells using Trypan blue exclusion

Granulosa cell viability and live cell numbers were determined by Trypan blue exclusion. The technique is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as Trypan blue, whereas dead cells do not. Therefore, when viewed under the microscope live cells appear as white/clear, while dead cells have a blue appearance. Briefly, 10 µl of the washed granulosa cell suspension is mixed with the appropriate volume of 0.4% Trypan blue (for example 10 µl in a 1:2 dilution) and incubated for 1 minute. Of this Trypan blue/granulosa cell mix solution 10 µl are applied to a haemocytometer. The number of live and dead cells is then counted in the 25 squares using a standard light microscope, which gives the proportion of live cells (viability), and the total number of live cells per ml of the cell preparation is calculated using the formula: (No. of live cells) x 10^4 x 2 (based on example given, or other dilution factor) Trypan Blue. This gives the total cell number per ml, if less or more than 1 ml is available before culture, or after culture following recovery of cells from wells, then this figure is multiplied by a volume correction factor to obtain the total number of cells present in the suspension.

2.10 Histology and Immunofluorescence

Quarter Ovary Collection and Cryosectioning

Following excision of the largest antral follicles from each ewe in year groups 1 and 3, the remaining ovarian tissue after follicular excision was quartered and a complete quarter unaffected by the excision process was either snap-frozen in liquid nitrogen (year group 1) or frozen in aluminium foil (year group 3) on dry ice and then stored at -80°C for subsequent histology and immunofluorescence.

All tissue sections were cut using a cryostat (Leica CM1850, Leica, Heidelberg, Germany). Quarter ovaries from year groups 1 and 3 were embedded onto appropriate sized cryocassettes using embedding material (OCT, Shandon, UK) and stored on the ultra-cold freezing bar until the embedding material was solid. The cryocassette was then attached to the cryostat cutting head and left for 1-2 hours to equilibrate the temperature between the cutting head and the ovary. The blade holder was set at an angle of 5°C and the temperature in the chamber was set at -23°C. This temperature was chosen following a period of optimisation to ascertain the optimal cutting conditions for ovarian tissue. Once sections were cutting freely, 14 μm serial sections were cut. Ten microscope slides were set-up on top of the cryostat at any one session. Serial sections were thaw mounted onto Polysine™ coated microscope slides (VWR International, Leicestershire, UK. Cat. No. 631-0107). When section 1 was cut it was thaw-mounted onto microscope slide number 1. Section 2 was mounted onto slide 2 and so on. Section 11 was then mounted back onto slide one and so on. It was possible to get at least 3 sections on one microscope slide. Once all 10 microscope slides were completed they were placed in a storage box in the chamber of the cryostat. Sections were cut through as much of the ovary quarter as was practically possible to ensure that the correct diameter of each antral follicle could be properly ascertained. Each quarter ovary produced between 100 and 270 sections. Following sectioning, all slides were stored at -80°C until processing.

Histological Processing and Characterization of ovaries

Every 10th frozen section from each quarter ovary was histologically stained with haematoxylin and eosin for follicle identification, sizing (diameter) and health determination as described in section 4.2.5. Frozen ovarian sections were fixed for 20 minutes in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer. Slides were then washed twice (5 minutes each) in 0.1 M phosphate Buffer, followed by a 3 minute wash in gently running tap water. Slides were then stained with haematoxylin for 5 minutes to stain the nucleus of cells, after which they were again washed for 3 minutes in gently running tap water. Sections were differentiated in acid-alcohol (0.5% (v/v) HCl in 70% (v/v) ethanol) for a few seconds, and then placed under gently running tap water (slightly alkaline) for 3 minutes. Sections were then counterstained with 1% (w/v) aqueous Eosin for 20 seconds. Slides were then passed up through a graded series of ethanol solutions to dehydrate the sections, being immersed for 5 seconds, 10 seconds and 2 x 3 minutes in 70, 90 and 100% (v/v) ethanol solutions, respectively. Once dehydrated, sections were finally washed twice (3 minutes each) in Histo-Clear®, after which the sections were coated in DPX mountant and cover-slips were attached.

Immunofluorescence

Every 10th frozen section from each quarter ovary was utilised for immunofluorescence to determine the presence or absence of the active form of Caspase 3 (CASP3) and 3-beta hydroxysteroid dehydrogenase (HSD3B1) in granulosa and theca cells, and aromatase (CYP19A1) in granulosa cells of antral follicles. Antigen retrieval was not required for any of the antibodies used. Frozen ovarian sections were quickly thawed and fixed for 20 minutes in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer. Slides were then washed three times with 0.05 M (pH 7.6) Tris buffered saline (TBS). To eliminate non-specific binding sites, sections were incubated with 10% (v/v) normal goat serum for 20 minutes at room temperature before incubating with primary antibodies for 2 hours at 4°C. Primary antibodies were against (1) CASP3 (rabbit polyclonal against activated form of human/mouse CASP3; AF835, R&D Systems, Minneapolis, MIN, USA), (2) HSD3B1 (rabbit polyclonal antibody against human recombinant type I HSD3B; kindly supplied by Dr. J.I. Mason, University of Edinburgh; validated for sheep gonads (Quirke et al., 2001) and (3) CYP19A1 (mouse monoclonal antibody against human CYP19A1; MCA1974T, AbD Serotec, Oxford, UK). Primary antibodies were diluted 1:500 (CASP3), 1:1000 (HSD3B1) and 1:250 (CYP19A1) in TBS/0.3% Triton/0.25% BSA. Negative control sections were incubated in carrier fluid only (TBS/0.3% Triton/0.25% BSA). After washing for 3 x 5 minutes in TBS, sections were incubated with fluorescently labelled secondary antibody for 45 minutes. The secondary antibodies used were goat anti-rabbit IgG (AlexaFluor® 488 F(ab)2 fragment; Invitrogen (Molecular Probes), Paisley, UK) for CASP3 and HSD3B1, and goat anti-mouse IgG (AlexaFluor® 594 F(ab)2 fragment; Invitrogen (Molecular Probes), Paisley, UK) for CYP19A1. Sections were coverslipped using FluorSave™ Reagent (Merck Chemical Ltd, Nottingham, UK), sealed using nail varnish and then viewed under a fluorescent microscope (Leica DM4000, Leica Microsystems (UK) Ltd., Milton Keynes, UK).

2.11 Statistical Analyses

Results were log10 transformed when normality tests using the raw data failed. For all parameters, log10 transformation was sufficient for data to pass normality teats. All analyses were carried out using Minitab (Minitab 15, Coventry, UK) or Stata™ (Stata version 10, StataCorp, College Station, Texas, USA).

A Fishers exact test was used to determine the differences in the proportion of any variable (e.g. percentage of ewes able to cycle in the first breeding season).

When only one factor was included in a statistical test (e.g. the effect of prenatal treatment on total ovarian weight when data were combined over all 5 years) a one way ANOVA was implemented and a Tukey's post-hoc test was used to determine any differences between levels within the one factor. For some data, if two factors were included (e.g. effects of prenatal treatment or year group on total ovarian weight) a two way ANOVA was used and Tukey's post-hoc test was used to determine any differences between levels within the one factor.

A general linear model (GLM) was used to analyse the data for chapter 3 to 5, when there were 2-3 factors (e.g. prenatal treatment, follicle classification, year group) that could influence dependent variables (e.g. follicular fluid diameter and steroid production). A GLM is as an extension of linear multiple regression for a single dependent variable. In multiple regression, more than one independent factor is used to predict the dependent variable. One way in which the general linear model differs from the multiple regression model is in terms of the number of factors that can be analyzed (multi-level modeling). A GLM allows random factors in addition to fixed factors to be included in a model unlike ANOVAs and multiple regression models. A P value of less than 0.05 was considered statistically significant for all types of analyses.

3.1 INTRODUCTION

Evidence accrued from previous studies has demonstrated that TP treatment *in utero* during the critical period of sexual differentiation and gonadal development leads to a raft of reproductive disruptions, at both the neuroendocrine and ovarian level (Sarma *et al.*, 2005; Steckler *et al.*, 2005; Unsworth *et al.*, 2005; Manikkam *et al.*, 2006; Padmanabhan *et al.*, 2006; Steckler *et al.*, 2007a; Smith *et al.*, 2008). While the breed of sheep used and individual variation appear to determine the severity of the phenotype, prenatal TP treatment of Suffolk (Manikkam *et al.*, 2006; Veiga-Lopez *et al.*, 2008) and Dorset (Birch *et al.*, 2003; Unsworth *et al.*, 2005) ewes results in the progressive loss of reproductive cycles, due to anovulation. Persistency of large follicles with elevated systemic oestradiol concentrations have been described in the Suffolk model, which demonstrates a less severe phenotype, with normal ovulations detected (Sarma *et al.*, 2005; Steckler *et al.*, 2008; Veiga-Lopez *et al.*, 2008). However, no group has investigated systemic oestradiol concentrations or large follicle development in prenatally TP-treated Dorset ewes, in which exogenous oestradiol is not able to generate gonadotrophin surges (Unsworth *et al.*, 2005) although progesterone elevations in the breeding season, in some cases followed by prolonged periods of elevated progesterone, appear to indicate some ovulatory activity (Unsworth *et al.*, 2005). This would provide further insights into the abnormal programming of ovarian and specifically follicular steroidogenesis by androgen excess.

Recent studies into the reproductive dysfunction of prenatally TP-treated ewes have focused on neuroendocrine, growth and metabolic disruptions (Birch *et al.*, 2003; Manikkam *et al.*, 2004; Unsworth *et al.*, 2005). At the ovarian level, follicular studies have concentrated on recruitment of primordial follicles and preantral follicle development (Steckler *et al.*, 2005; Smith *et al.*, 2008) or on the morphological description of large antral follicle growth using ovarian ultrasound (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). Androgenisation by TP but not DHT, which is considered to be a non-aromatisable androgen, resulted in follicular persistence with lack of dominance as more and more follicles failed to regress in Suffolk ewes (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a), providing evidence that follicle growth and atresia in adult ewes are altered by the *in utero* programming actions of oestrogen. As follicle persistence or loss of dominance during normal or manipulated ruminant cycles are related to alterations in follicular steroid synthesis (Mihm *et al.*, 1999), it is conceivable that abnormal programming of follicular

steroidogenesis occurs in individual large antral follicles due to TP excess *in utero*, and this, to the best of our knowledge, has not yet been investigated. Furthermore, while a lot of information has been gathered relative to the abnormal programming of neuroendocrine events and systemic ovarian function by prenatal TP excess, the relative roles of oestrogen and androgen in programming specifically follicular dysfunctions, particularly within the largest antral follicles, remain to be determined.

The main aim of this study was to determine how individual large antral follicle function, specifically growth and steroidogenesis is altered in prenatally TP- and DHT-treated Dorset ewes. Diameter, intrafollicular oestradiol, progesterone and testosterone concentrations and the oestradiol to progesterone ratio were the follicle parameters investigated in the two largest follicles per animal, classified into the dominant follicle (DF) with highest intrafollicular oestradiol and the largest subordinate follicle (SF). For the first time, peripheral oestradiol concentrations were investigated in and out of the breeding season to provide more insights into the programming of follicular steroidogenesis by TP. Additionally, the study aimed to confirm previous observations in the prenatally TP-treated ewe, with regards to increased ovarian weight and altered reproductive cycles determined from progesterone profiles (West *et al.*, 2001; Birch *et al.*, 2003; Unsworth *et al.*, 2005). Finally, this study is the first to investigate the consistent effects of prenatal androgenisation by TP (and not DHT) over several year groups within the same study, instead of studying only one group of animals.

3.2 Materials and Methods

3.2.1 Animals and Prenatal Treatment

Poll Dorset ewes were androgenised *in utero* using an established model (Robinson *et al.*, 1999; Steckler *et al.*, 2005) and euthanased at 10- (Year Group 3, Control = 7, TP = 10, Year 4, Control = 7, TP = 6 and Year Group 5, Control = 14, TP = 15, DHT = 8), 12- (Year Group 2, Control = 11, TP = 7 ewes) and 22-months of age (Year Group 1, Control = 9, TP = 7) for ovary collection. Reproductive cycles were not synchronised at any stage throughout the study.

3.2.2 Collection of blood samples

Plasma samples were collected by jugular venipuncture into heparinised tubes from control and androgenised ewe lambs twice weekly from 25 weeks of age (puberty ~30 weeks Foster, 1994) until about a month after the expected end of the breeding season in all five groups of sheep (Section 2.3). All samples were assayed for progesterone to determine ovulatory cycles, while a subset of samples were selected for the determination of

oestradiol concentrations (two plasma samples were taken three days apart at the beginning (December), after the end of the breeding season (February) and in year group 1 only, in the middle of June (anoestrous). Following collection, samples were stored at –20°C until required for assay.

3.2.3 Collection of tissues

Ovaries from control, TP and DHT-treated females were harvested at 10- and 22-months of age and each ovary was weighed following recovery. Following this, ovarian maps were drawn for each ovary and in the majority of ewes the largest (generally two) antral follicles were identified from surface measurements using callipers, excised and the follicular fluid collected as described in section 2.4. In order to be excised, follicles had to be greater than 3.5 mm in diameter in control and DHT-treated ewes, and a minimum of 5 mm in diameter in TP-treated ewes. In 6/35 control, 2/8 DHT animals and 7/37 TP ewes only one follicle fulfilled the size criteria and was thus excised. In two TP-treated ewes from year group 4 three follicles fulfilled the size criteria, and all three were excised as it was not possible to distinguish the largest two by diameter. Of the similarly sized follicles the two with highest intrafollicular oestradiol were used in the study. Prior to follicular fluid collection, individually excised follicles were again measured by callipers to accurately determine their diameter.

3.2.4 Hormone Assays

Concentrations of the steroid hormones oestradiol and progesterone in plasma and follicular fluid of the largest antral follicles were determined using validated radioimmunoassays (RIA) (plasma oestradiol (Evans *et al.*, 1994), plasma progesterone (Padmanabhan *et al.*, 1995), follicular fluid oestradiol and progesterone (Evans & Martin, 2000). Testosterone concentration was determined only in follicular fluid. All follicular fluid samples were diluted in MAIA buffer (0.01 M PBS, 0.1% gelatin, and 0.1% sodium azide, pH 7.0). Follicular fluid samples were diluted as detailed in section 2.6 and required no prior extraction. Two hundred µl of plasma from ewes of year groups 1-4 were extracted with diethylether (Rathburn Chemicals Ltd., Walkerburn, Scotland, UK) in duplicate to determine oestradiol concentrations in plasma samples. No prior extraction was required for determination of progesterone concentrations in plasma. The limit of assay sensitivity was assigned to those samples in which the concentration of oestradiol, progesterone or testosterone was below the sensitivity of the assay despite the maximum volume available added to the assay. Pooled follicular fluid and plasma samples were used in each assay as quality controls. The gamma counter used was a Cobra™ II Auto-Gamma®

(Packard Bioscience, UK) and the liquid scintillation analyzer, 1600TR liquid scintillation analyzer (Packard Bioscience, UK).

Oestradiol RIA

Plasma and follicular fluid oestradiol concentrations were determined using a commercial RIA kit (MAIA Oestradiol Kit, Bio-Stat Limited., Stockport, UK. Cat no. 370001). Plasma oestradiol concentrations per ewe at the start (December) and after the end of the breeding season (February) were established by averaging the two samples taken three days apart. Samples were run for each year group (1-4) in one assay per year group (n = 4 assays). Mean intra-assay coefficient of variation for one quality control sample (per year group) at a concentration of 1.03, 1.02, 1.18 and 1.45 pg/ml was 8.9%, 9.1%, 9.7% and 9.3% for sheep year groups 1, 2, 3 and 4, respectively. Sensitivity of the assay, calculated as two standard deviations below the mean counts per minute (CPM) at maximum binding, was 0.25 ± 0.03 pg/ml. Extraction efficiency, determined by spiking samples with iodine labelled estradiol followed by extraction by diethyl ether and finally counting the remaining iodinated estradiol was calculated as 78%. However, standards were also extracted, thus levels of oestradiol in plasma should not have been underestimated.

Follicular fluid samples were assayed in duplicate for year groups 2-5 over two assays per year group (n = 8 assays). Mean intra-assay coefficient of variation for one quality control sample at a concentration of 12.25, 14.16, 17.18 and 13.45 ng/ml was 4.6%, 5.1%, 4.6% and 4.8% for sheep year groups 2, 3, 4 and 5, respectively. Mean inter-assay coefficient of variation for one quality control sample (for each year group) was 6.9%, 6.1%, 6.6% and 7.8% for sheep year groups 2, 3, 4 and 5 respectively. Sensitivity of the assay, calculated as two standard deviations below the mean counts per minute (CPM) at maximum binding, was 3.5 ± 0.50 pg/ml.

Progesterone RIA

Progesterone concentrations were measured in plasma samples from ewes in year groups 1-5 by RIA using a Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA) previously validated for use in sheep (Padmanabhan *et al.*, 1995). Elevations in progesterone concentrations in blood samples were used to indicate reproductive cycles, such that a cycle was determined to have occurred if progesterone concentrations were raised to greater than 1 ng/ml for two but no more than three samples (therefore, spanning at least 10 but no more than 14 days; normal length of the ovine luteal phase) before decreasing to values less than 1 ng/ml for at least one sample (normal length of the follicular phase) before the onset of the following cycle (Birch *et al.*, 2003). Ewes were

defined as in the breeding season if control ewes displayed a progesterone cycle at the time of sample collection.

Samples were run in one assay for each year group (n = 5 assays). Mean intra-assay coefficient of variation for one quality control sample at a concentration of 0.65, 0.67, 0.85, 0.56 and 0.55 ng/ml was 8.8%, 9.5%, 9.1%, 8.7% and 8.3% for sheep year groups 1, 2, 3, 4 and 5, respectively. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 0.23 ± 0.03 ng/ml.

Follicular fluid samples were assayed in duplicate for year groups 2-5 over two assays per year group (n = 8 assays). Mean intra-assay coefficient of variation for one quality control sample at a concentration of 23.4, 35.5, 27.2 and 33.2 ng/ml was 6.3%, 7.7%, 6.9% and 7.9% for year groups 2, 3, 4 and 5 respectively. Mean inter-assay coefficient of variation for one quality control sample (for each year group) was 8.7%, 8.8%, 9.1% and 9.5% for sheep year groups 2, 3, 4 and 5, respectively. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 0.8 ± 0.06 ng/ml.

Follicular Fluid Testosterone RIA

Follicular fluid concentrations of testosterone were measured without prior extraction using an established assay (Sheffield & O'Shaughnessy, 1989). The primary antibody (rabbit antiserum) (GE Healthcare, Amersham Place, Buckinghamshire, UK), was diluted to 1:900 in MAIA buffer.

Testosterone concentrations were determined over two assays. Mean intra- and inter-assay coefficients of variation for one quality control sample at a concentration of 56.5 pg/ml were 9% and 13% respectively and the sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 10.23 ± 0.04 pg/ml.

3.2.5 Statistical Analyses

A Fisher's exact test was utilised to determine any differences in the percentage of ewes cycling in the first breeding between prenatal treatment (control, TP and DHT) groups. A Fishers exact test was used to determine any differences in the proportion of ewe lambs cycling in the first breeding season when data was combined over all 5 years. A General Linear Model (GLM), with year group (1-4), prenatal treatment (Control, TP) and sample time-point (December, February and June) as the fixed factors was applied to determine any influences on circulating concentrations of oestradiol. A GLM, with prenatal treatment (Control, TP and DHT) and year group (1-5) as the fixed factors was applied to determine any differences in ovarian weight. A one-way ANOVA was used to determine any differences in ovarian weight when data was combined over all 5 years. If there were more

than 2 levels to a factor, a Tukey's post hoc test was used to determine significant differences between levels within that factor.

For individual follicle parameters (follicle size, follicular fluid oestradiol, progesterone and oestradiol: progesterone ratio) a GLM with year group (2-5), prenatal treatment (Control, TP, and DHT) and follicle classification (DF - follicle with highest intrafollicular oestradiol, and generally but not always the largest follicle, and SF - the second of the two largest follicles recovered but with lower intrafollicular oestradiol than the DF) as the fixed factors was used to determine any influences on follicle function. In addition, any logical interactions between factors were included in the model. Because the factor 'animal' is known to contribute to the variation in results, but could not be included in the GLM analyses, a generalised least square (GLS) model was also applied to the data with the same fixed factors as for the GLM analyses, but with 'animal' included as a random factor to determine whether this altered the outcome of GLM analyses. However, the GLS analysis did not consider interactions and only had limited post hoc analyses. Because of this and the good agreement between GLM and GLS analyses, the results from the GLM analyses are presented. If there were more than 2 levels to a factor, a Tukey's post hoc test was used to determine significant differences between levels within that factor.
A two way ANOVA with follicle classification (DF and SF) and prenatal treatment (control and TP) as the factors was used to determine any influences on follicular fluid testosterone concentrations.

3.3 Results

3.3.1 Reproductive Cycles in the First Breeding Season

Representative plasma progesterone profiles from control, TP and DHT-treated ewes are shown in Figure 3.1. The proportion of ewes undergoing at least one reproductive cycle during the first breeding season in year groups 1 to 5 is shown in Figure 3.2. Despite consistent absolute reductions in (TP) androgenised ewes from year groups 1, 2, 3 and 5, there was no difference between treatment groups in the proportion of ewes cycling in the first breeding season when results were analysed separately for each year. Data were combined across the 5 year groups of sheep for analysis and the proportion of ewes demonstrating at least one reproductive cycle in the breeding season was significantly reduced ($P < 0.05$) by prenatal androgenisation (Figure 3.3).

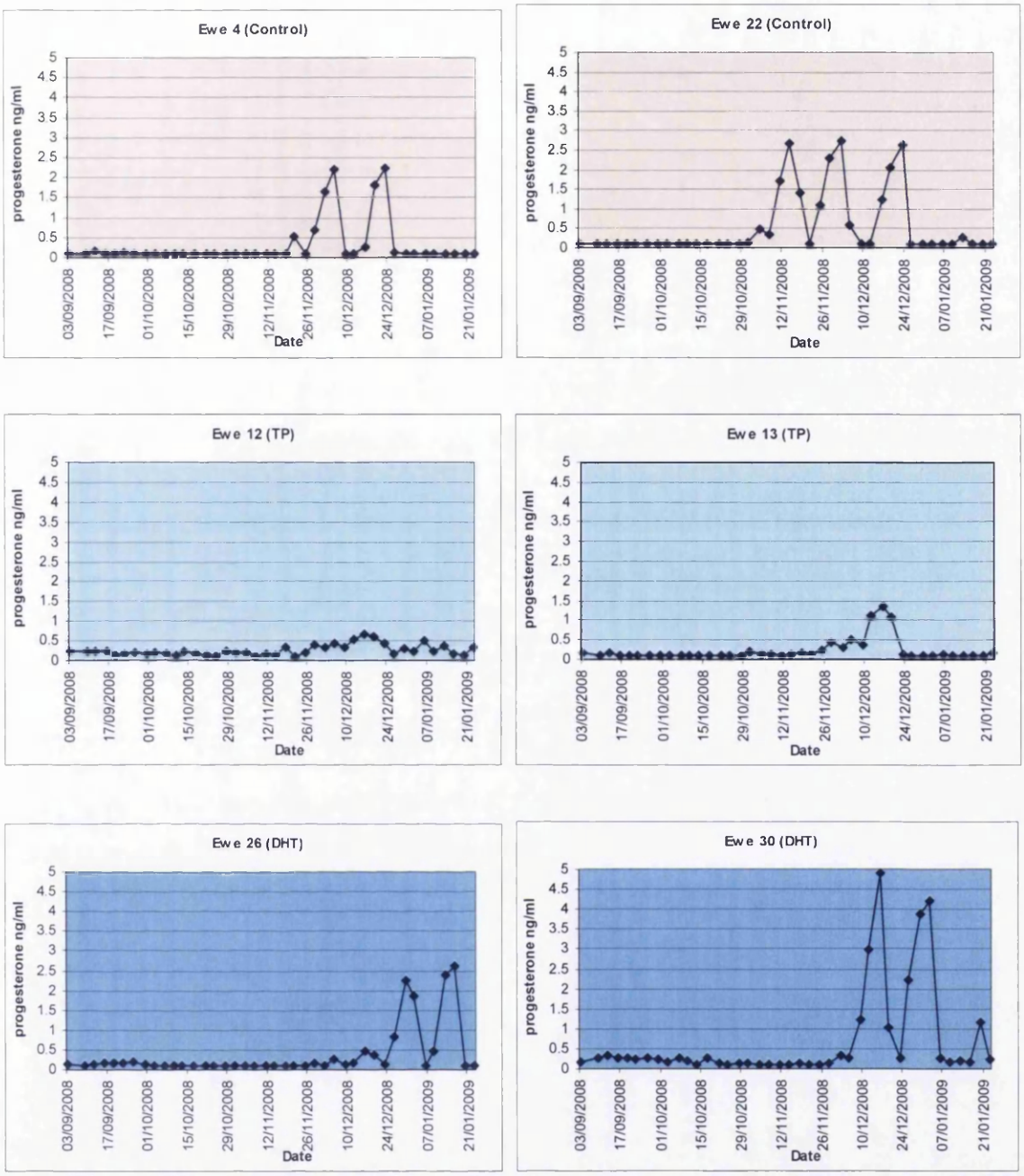


Figure 3.1 Plasma progesterone profiles from representative control, testosterone propionate (TP) and 5α-dihydrotestosterone (DHT) treated ewes from year group 5. Blood samples were taken twice weekly from early September (the expected onset of puberty (~30 weeks) to the end of January (end of the first breeding season).

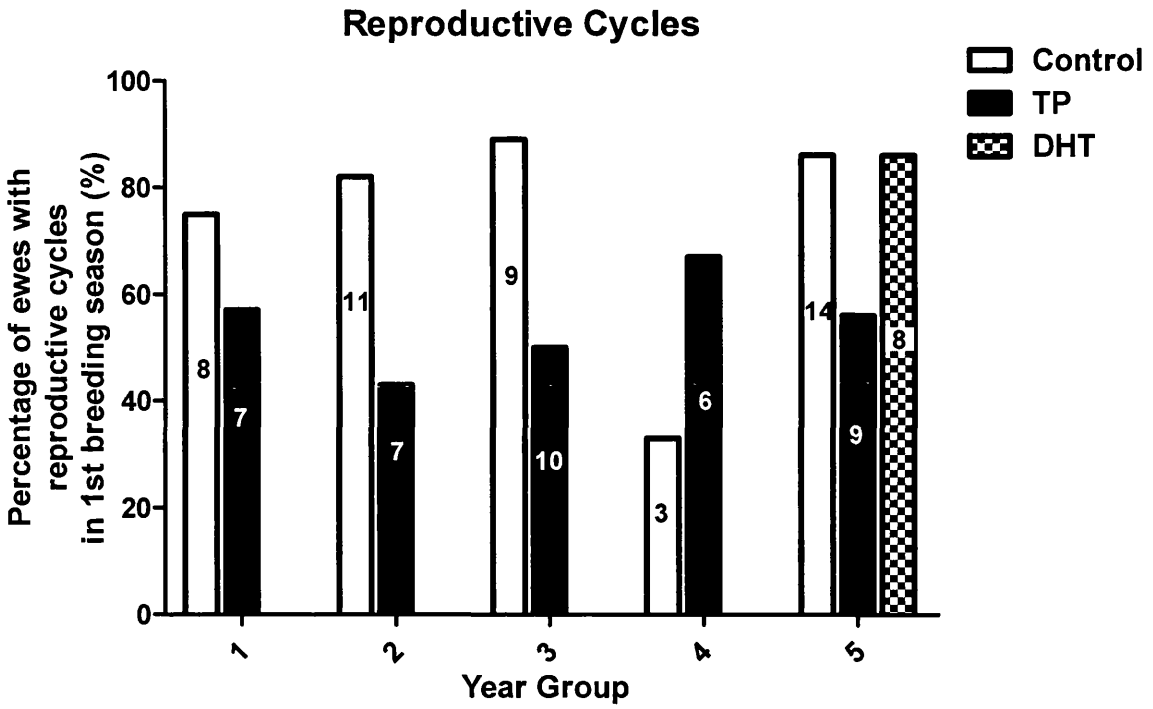


Figure 3.2 Effect of prenatal androgen (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) treatment on the proportion of sheep with reproductive cycles in the first breeding season. The total number of ewes within each year and treatment group are shown inside each column on the bar graph.

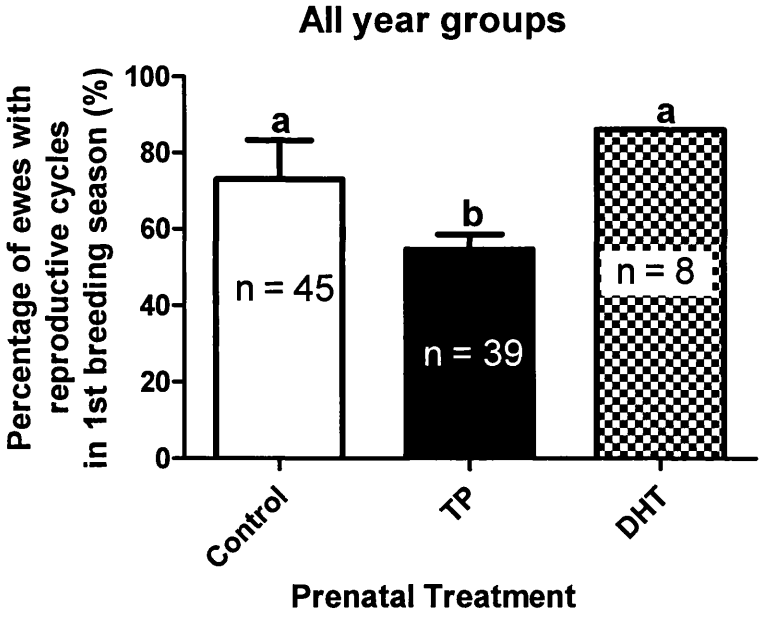


Figure 3.3 Effect of prenatal androgen (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) treatment on the proportion of sheep with reproductive cycles in the first breeding season when data is combined over the 5 year groups. [a,b] Within a treatment group, means without a common superscript differ ($P < 0.05$).

3.3.2 Circulating Oestradiol Concentration

A summary of the circulating oestradiol concentrations measured in ewes from year groups 1-4 is shown in Table 3.1. Peripheral concentrations were elevated in TP-treated ewes ($P < 0.001$) from year group 1 sheep compared with control ewes when samples were collected in June (animals were 14 months old and out of the breeding season). Peripheral oestradiol concentrations were also elevated in TP-treated ewes from 10 month old sheep from year groups 3 and 4, when samples were taken in February (just after the end of the breeding season) ($P < 0.001$ and < 0.05 respectively). Peripheral oestradiol concentrations were increased in TP ewes aged 8 months (year group 4), when sheep were in the breeding season. No significant difference in circulating concentrations of oestradiol between the two treatment groups (C and TP) was found in year groups 2 and 3 when samples were collected in December during the middle of the first breeding season.

Age of ewe at time of sample (year group)	Month samples taken	In the breeding season?	Control plasma oestradiol concentration (pg/ml)	TP plasma oestradiol concentration (pg/ml)	P value
14 months (1)	June	No	0.62 ± 0.13 (n=8)	1.39 ± 0.11 (n=7)	<0.001
8 months (2)	December	Yes	0.61 ± 0.09 (n=8)	0.73 ± 0.14 (n=7)	0.675
8 months (3)	December	Yes	1.54 ± 0.42 (n=9)	2.7 ± 0.62 (n=10)	0.112
10 months (3)	February	No	0.29 ± 0.06 (n=9)	0.99 ± 0.2 (n=10)	<0.001
8 months (4)	December	Yes	0.48 ± 0.16 (n=3)	2.01 ± 0.66 (n=6)	0.0243
10 months (4)	February	No	0.33 ± 0.01 (n=3)	1.44 ± 0.24 (n=6)	0.0354

Table 3.1 Peripheral oestradiol concentrations in control and prenatally TP-treated ewes from year groups 1-4, in (December samples) or out of the breeding season (February samples). The average concentration of two samples taken three days apart in the month was used to determine peripheral oestradiol concentrations. TP = testosterone propionate. Values presented are mean ± SEM.

3.3.3 Ovarian Weight

Ovaries recovered from ewes treated prenatally with TP were significantly heavier in year group 3 ($P < 0.01$) and year group 5 ($P < 0.001$) in comparison to control ewes (Figure 3.4). There was no significant difference in ovarian weight between control and TP-treated ewes in year groups 1, 2 and 4. Ovaries recovered from ewes treated prenatally with DHT in year group 5 were lighter in comparison to TP-treated ewes (ANOVA, $P < 0.05$), however, there was no significant difference in ovarian weight between the DHT and controls groups. In addition, year group 1 control ovaries, recovered from ewes 12 months older than ewes from the other year groups, were heavier compared with year group 2, 3 and 5 control ovaries. Overall, androgenisation by TP significantly ($P < 0.05$) increased total ovarian weight compared to both control and DHT-treated groups when data were combined from all the year groups (Figure 3.5).

3.3.4 Individual Large Antral Follicles

3.3.4.1 Follicle Diameter

The diameter of DF and SF recovered from control and androgenised ewes is shown in Figure 3.6. Prenatal androgenisation by TP increased follicle diameter of the largest follicles compared to controls. Androgenisation by DHT led to intermediate follicle diameters, which did not differ from diameters measured in control or TP-treated ewes. There was no overall difference in follicle diameter between follicles classified as DF or SF (Figure 3.11) However, an interaction between prenatal treatment and follicle classification was determined, as DF recovered from TP-treated ewes only were larger than SF recovered from the same ewe (Figure 3.12). There was no difference between year groups in the follicle diameter of DF and SF recovered.

3.3.4.2 Follicular Fluid Oestradiol Concentration

Follicular fluid oestradiol concentrations within DF and SF recovered from control and androgenised ewes are shown in Figure 3.7. Prenatal androgenisation by TP increased follicular fluid oestradiol concentration overall in both DF and SF compared with follicles from control and DHT-treated animals. Androgenisation by DHT had no effect on follicular fluid oestradiol concentration compared to controls. Follicular fluid oestradiol concentrations were also increased in DF versus SF (Figure 3.11), which confirmed the follicle classification and suggests a higher health status in the DF (follicle health being linked with intrafollicular oestradiol production (Tetsuka & Nancarrow, 2007) compared with the SF within the same animal. There was no difference between year groups in the follicular fluid oestradiol concentration of DF and SF recovered.

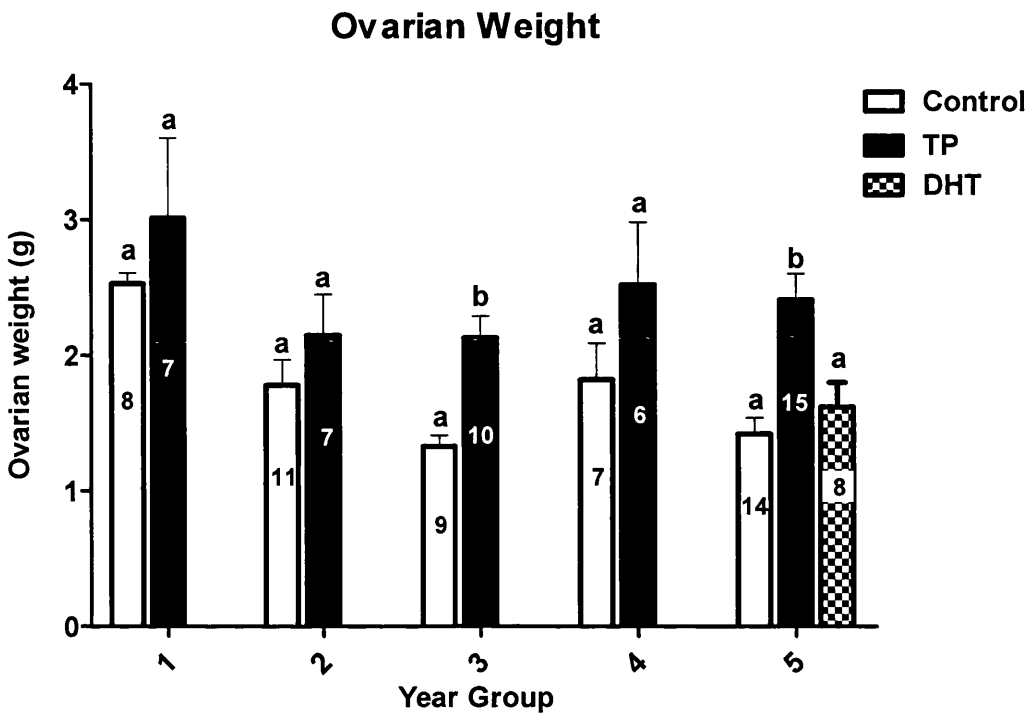


Figure 3.4 Effect of prenatal androgen (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) treatment on total ovarian weight in the five year groups of sheep studied. Values presented are mean ± SEM. (Group 1 – C = 8; TP = 6: Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 10: Group 4 – C = 7; TP= 6 ewes and Group 5 – C = 14, TP = 15 and DHT = 8). [a,b] Within a year group, means without a common superscript differ ($P < 0.05$).

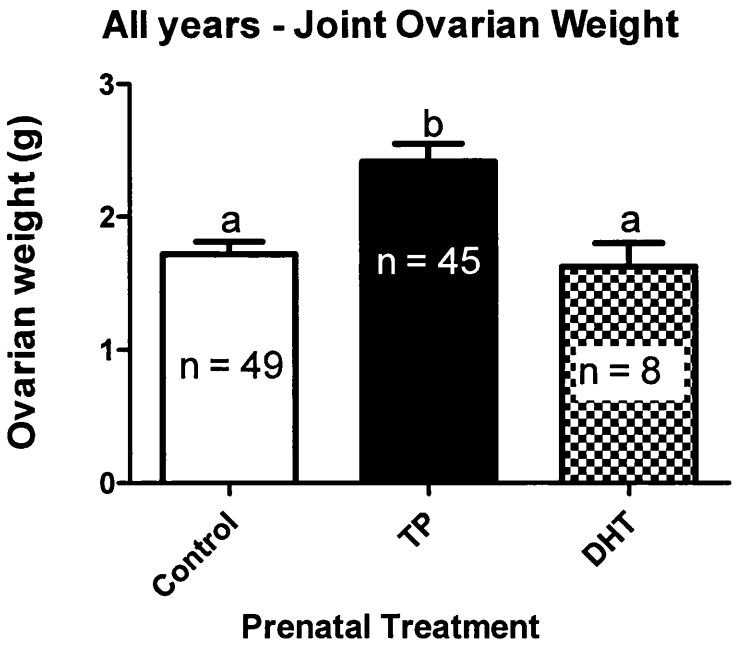


Figure 3.5 Effect of prenatal androgen (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) treatment on total ovarian weight when weights are combined across the 5 year groups used in the study. Values presented are mean ± SEM. [a,b] Means without a common superscript differ ($P < 0.05$).

3.3.4.3 Follicular Fluid Progesterone Concentration

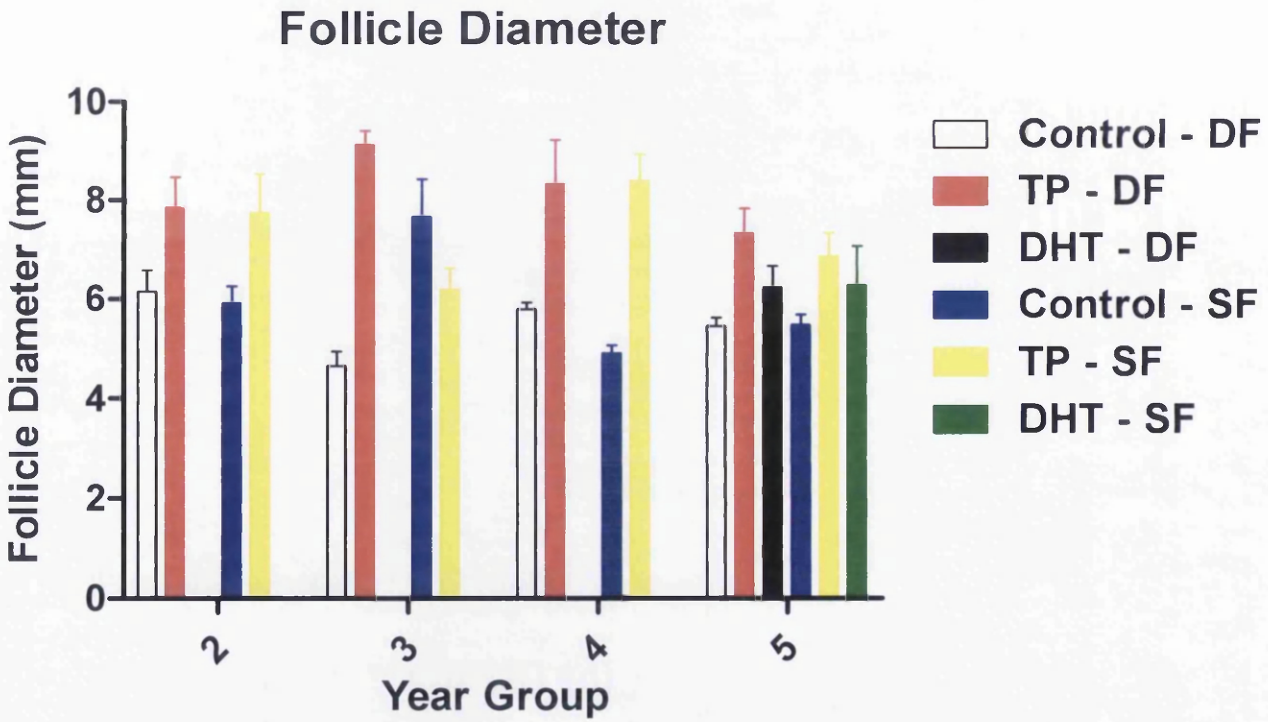
Follicular fluid progesterone concentrations within DF and SF recovered from control and androgenised ewes are shown in Figure 3.8. Prenatal androgenisation by TP increased follicular fluid progesterone concentration overall in both DF and SF compared with follicles from control and DHT-treated animals. Androgenisation by DHT had no effect on follicular fluid progesterone concentration compared to controls. Follicular fluid progesterone concentrations were similar between DF and SF (Figure 3.11). Follicular fluid progesterone differed between year groups, specifically intrafollicular progesterone concentrations were higher in year group 2 compared to 3.

3.3.4.4 Follicular Fluid Oestradiol to Progesterone Ratio

Prenatal androgenisation had no effect on the follicular fluid oestradiol to progesterone ratio (Figure 3.9). The oestradiol to progesterone ratio was increased in follicles classified as DF versus SF, confirming our functional classification (Figure 3.11). Follicular fluid oestradiol to progesterone ratio was lower in year groups 2 and 5 compared to year groups 3 and 4.

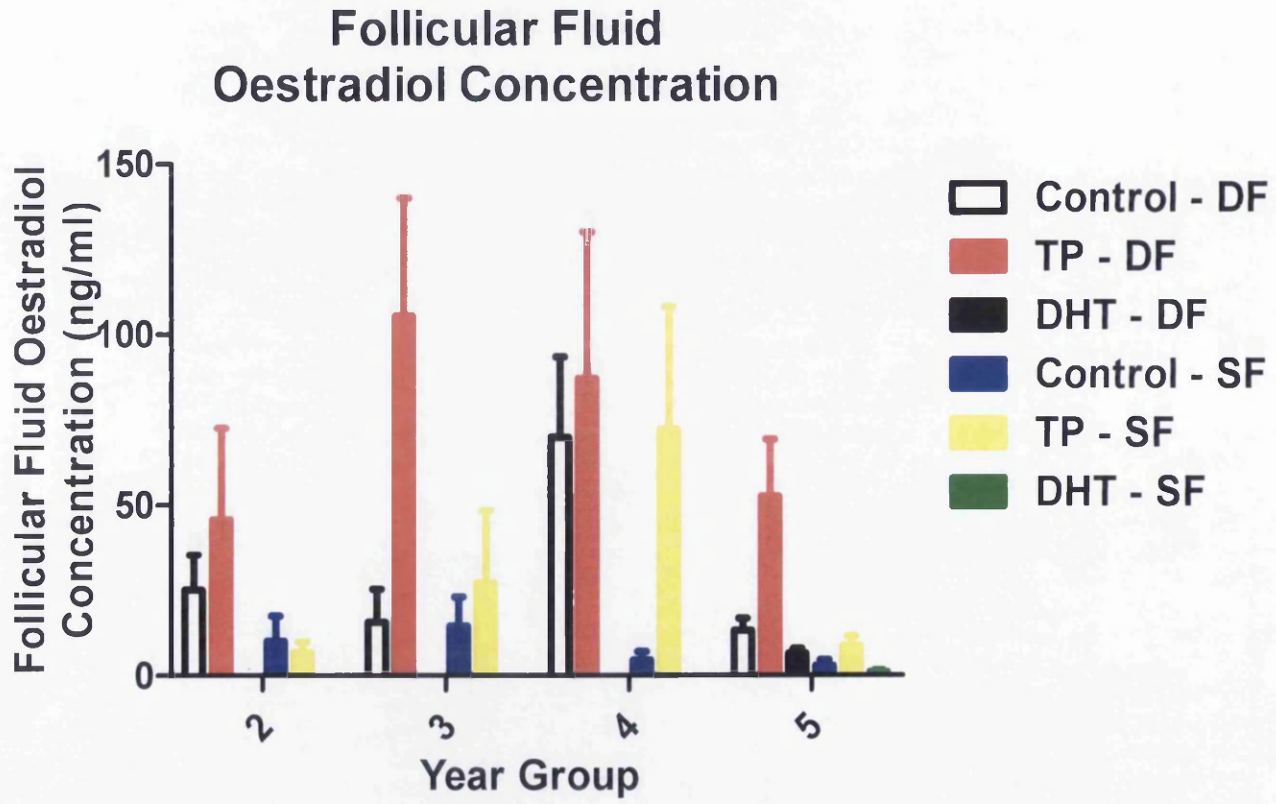
3.3.4.5 Follicular Fluid Testosterone Concentration

Follicular fluid testosterone concentrations from DF and SF recovered from control and androgenised ewes of year group 2 are shown in Figure 3.10. Prenatal androgenisation by TP had no effect on follicular fluid testosterone concentrations in DF and SF recovered from each ewe. Follicular fluid testosterone concentrations were increased in follicles classified as SF versus DF.



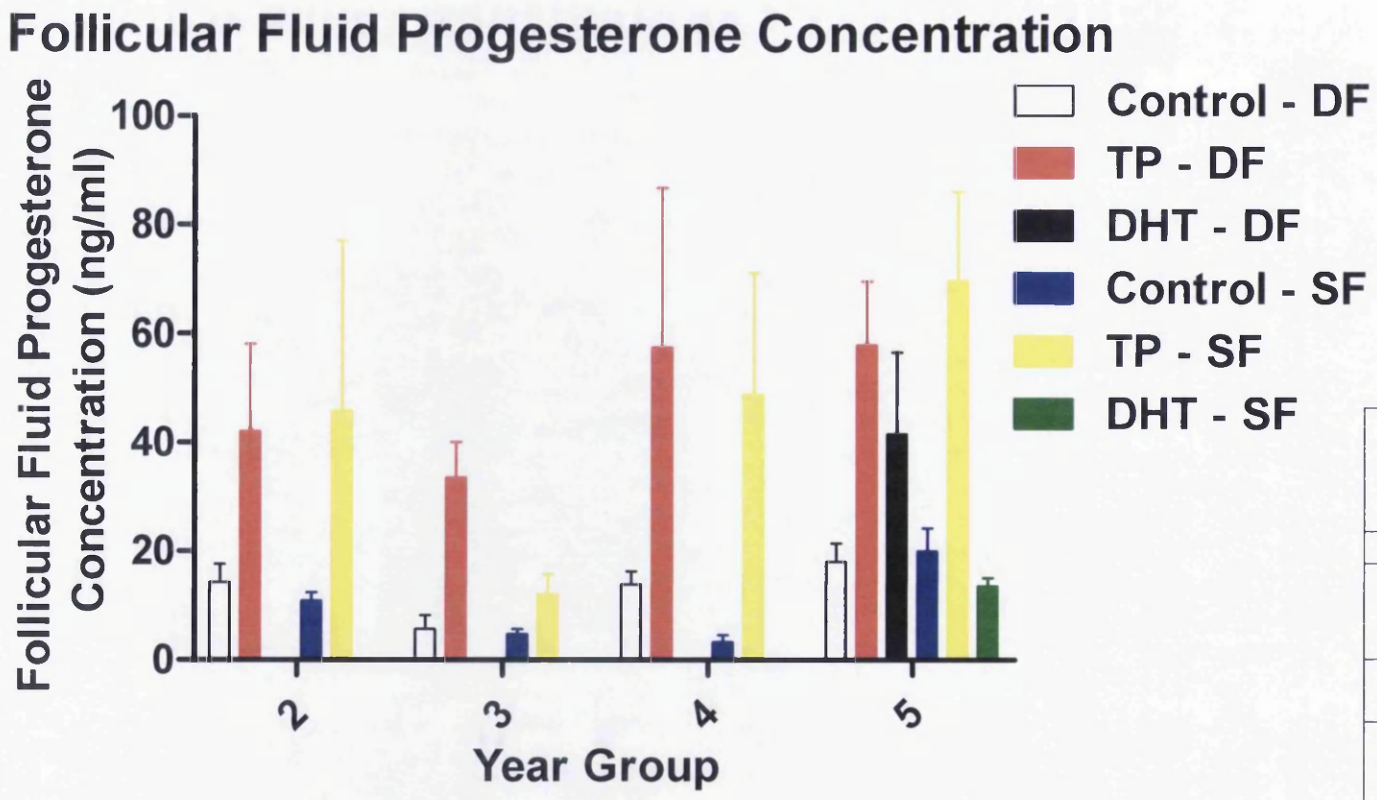
Factor	GLM P value	Comparisons of levels within factors	Tukey post-hoc P value
Year	0.561	N/A	N/A
Prenatal Treatment	0.000	C vs TP	0.002
		C vs DHT	0.225
		TP vs DHT	0.160
Follicle Classification	0.159	N/A	N/A
Prenatal Treatment*Follicle Classification	0.044	N/A	N/A

Figure 3.6 Effect of prenatal androgenisation (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) treatment on the diameter (size) of dominant (DF) and subordinate (SF) follicles. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 14; TP = 15; DHT DF = 8 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 12; TP = 13; DHT = 5 follicles). N/A – not applicable.



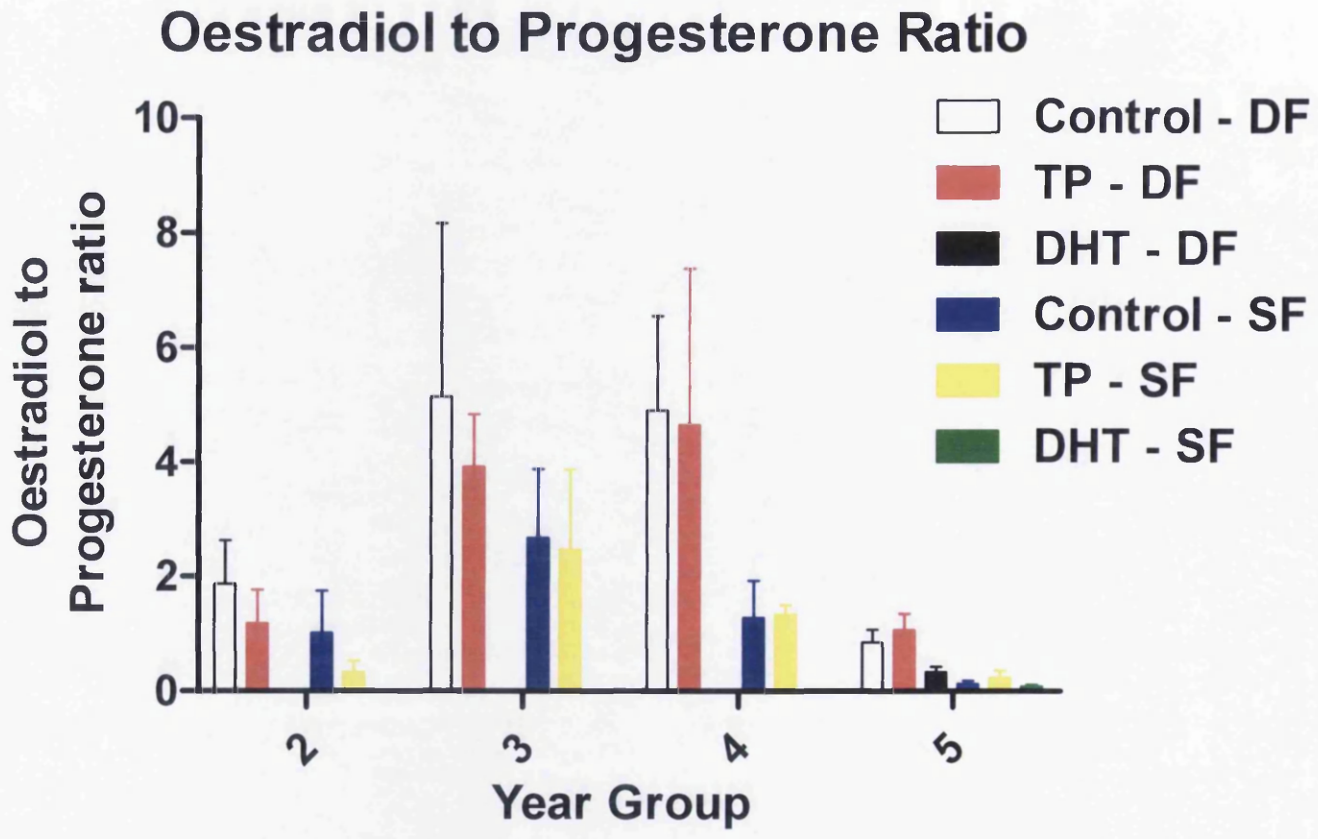
Factor	GLM P value	Comparisons of levels within factors	Tukey post-hoc P value
Year	0.251	N/A	N/A
Prenatal Treatment	0.007	C vs TP	0.002
		C vs DHT	0.65
		TP vs DHT	0.008
Follicle Classification	0.000	N/A	N/A
Prenatal Treatment*Follicle Classification	0.815	N/A	N/A

Figure 3.7 Effect of prenatal androgenisation (TP = testosterone propionate, DHT - 5α-dihydrotestosterone) on the follicular fluid oestradiol concentration of the dominant (DF) and subordinate (SF) follicles. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 14; TP = 15; DHT = 8 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 12; TP = 13; DHT = 5 follicles). N/A – not applicable.



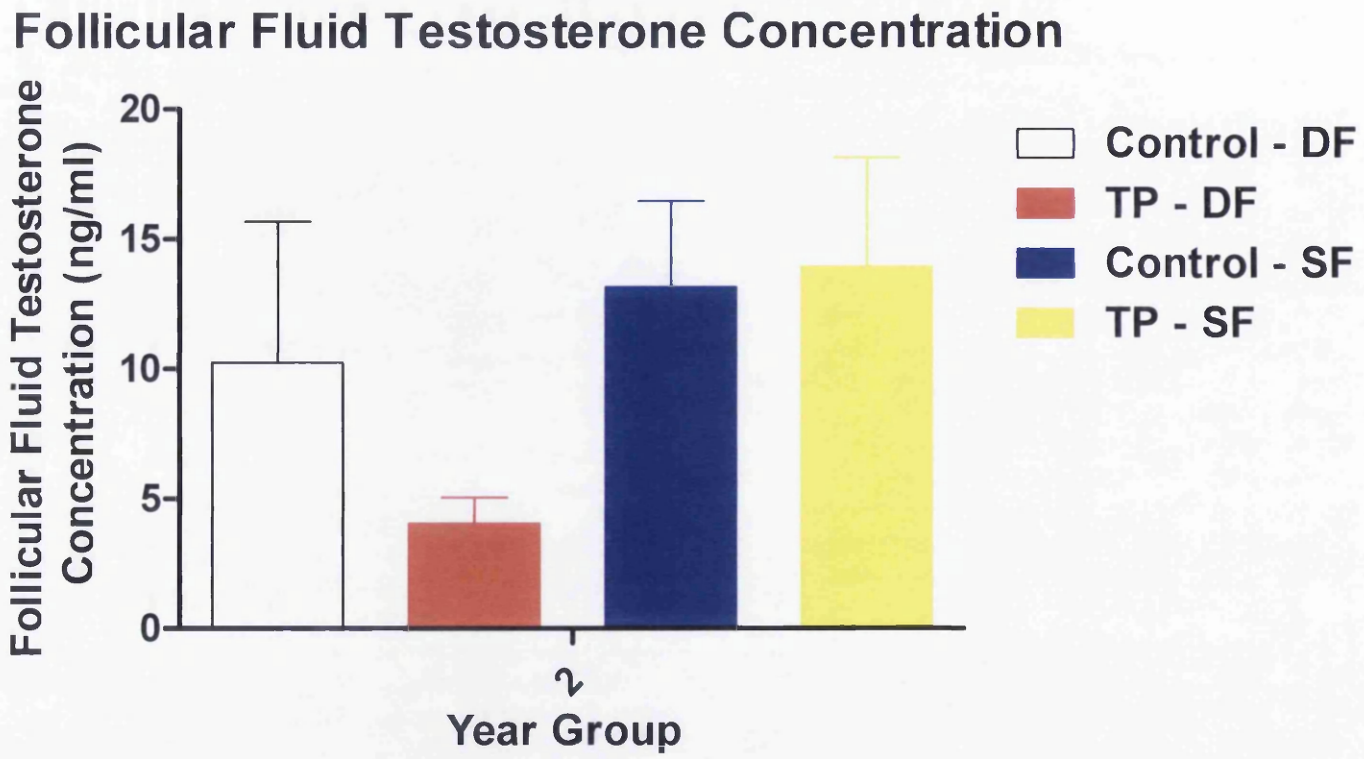
Factor	GLM P value	Comparisons of levels within factors	Tukey post-hoc P value
Year	0.000	2 vs 3	0.033
Prenatal Treatment	0.000	C vs TP	0.001
		C vs DHT	0.394
		TP vs DHT	0.050
Follicle Classification	0.174	N/A	N/A
Prenatal Treatment*Follicle Classification	0.278	N/A	N/A

Figure 3.8 Effect of prenatal androgenisation (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) on the follicular fluid progesterone concentration of dominant (DF) and subordinate (SF) follicles. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 14; TP = 15; DHT = 8 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 12; TP = 13; DHT = 5 follicles). N/A – not applicable.



Factor	GLM P value	Comparisons of levels within factors	Tukey post-hoc P value
Year	0.000	2 vs 3	0.023
		2 vs 4	0.021
		3 vs 5	0.011
		4 vs 5	0.024
Prenatal Treatment	0.657	N/A	N/A
Follicle Classification	0.000	N/A	N/A
Prenatal Treatment*Follicle Classification	0.857	N/A	N/A

Figure 3.9 Effect of prenatal androgenisation (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) on the follicular fluid oestradiol to progesterone ratio within dominant (DF) and subordinate (SF) follicles. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 14; TP = 15; DHT = 8 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 12; TP = 13; DHT = 5 follicles). N/A – not applicable.



Factor	GLM P value
Prenatal Treatment	0.56
Follicle Classification	0.013
Prenatal Treatment*Follicle Classification	0.58

Figure 3.10 Effect of prenatal androgenisation by TP (testosterone propionate) on the follicular fluid testosterone within dominant (DF) and subordinate (SF) follicles. Values presented are mean ± SEM. (DF = Year Group 2 – C = 7; TP = 6 - SF = Group 2 – C = 5; TP = 5 follicles).

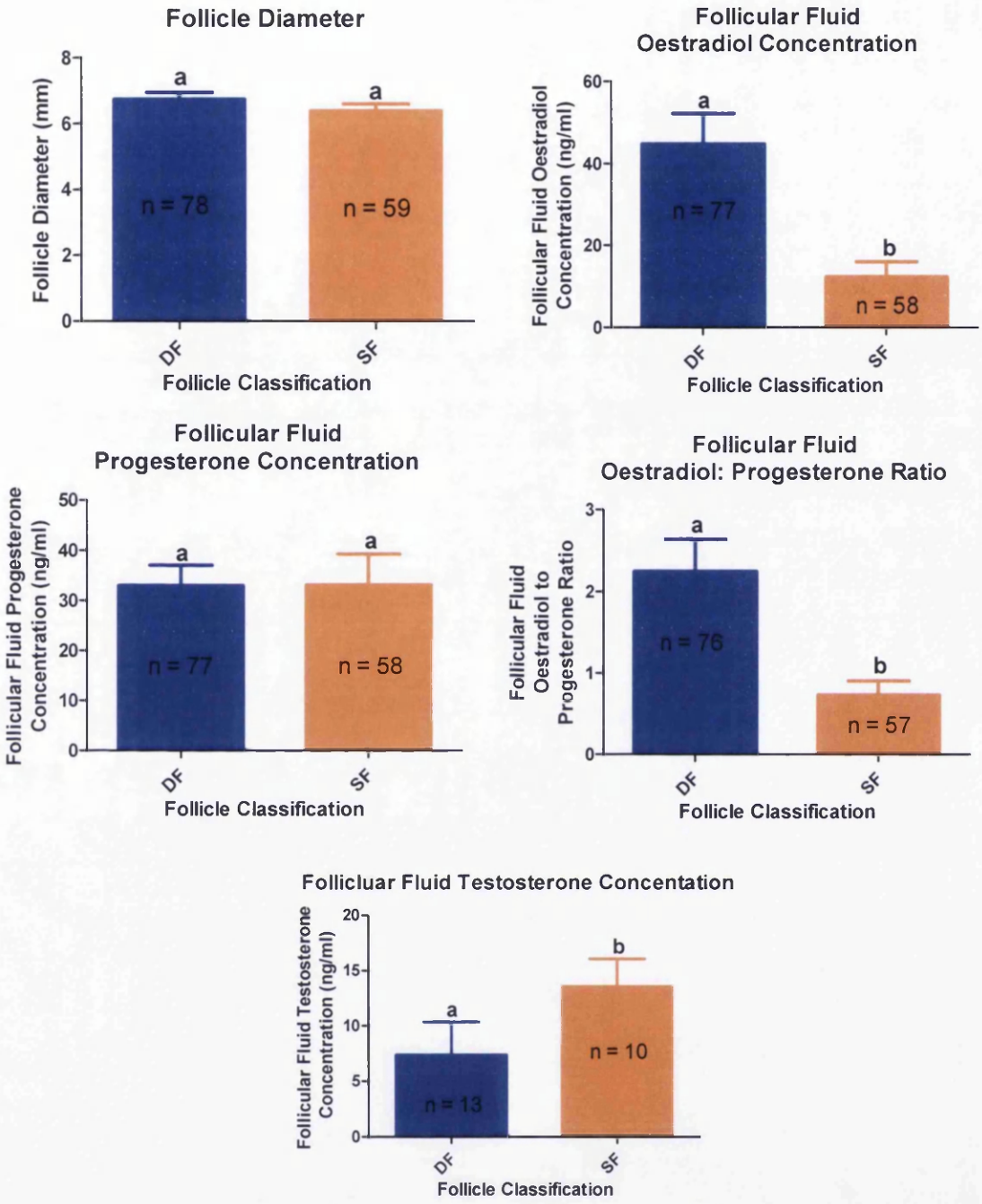


Figure 3.11 Intrafollicular characteristics (follicle diameter, follicular fluid oestradiol, progesterone, oestradiol to progesterone ratio and testosterone) of dominant (DF) and subordinate (SF) follicles when was combined across both treatment groups and all year groups. Values presented are Mean ± SEM. [a,b] Within a follicle classification, means without a common superscript differ ($P < 0.05$). Note that follicular fluid testosterone levels were only investigated in year group 2.

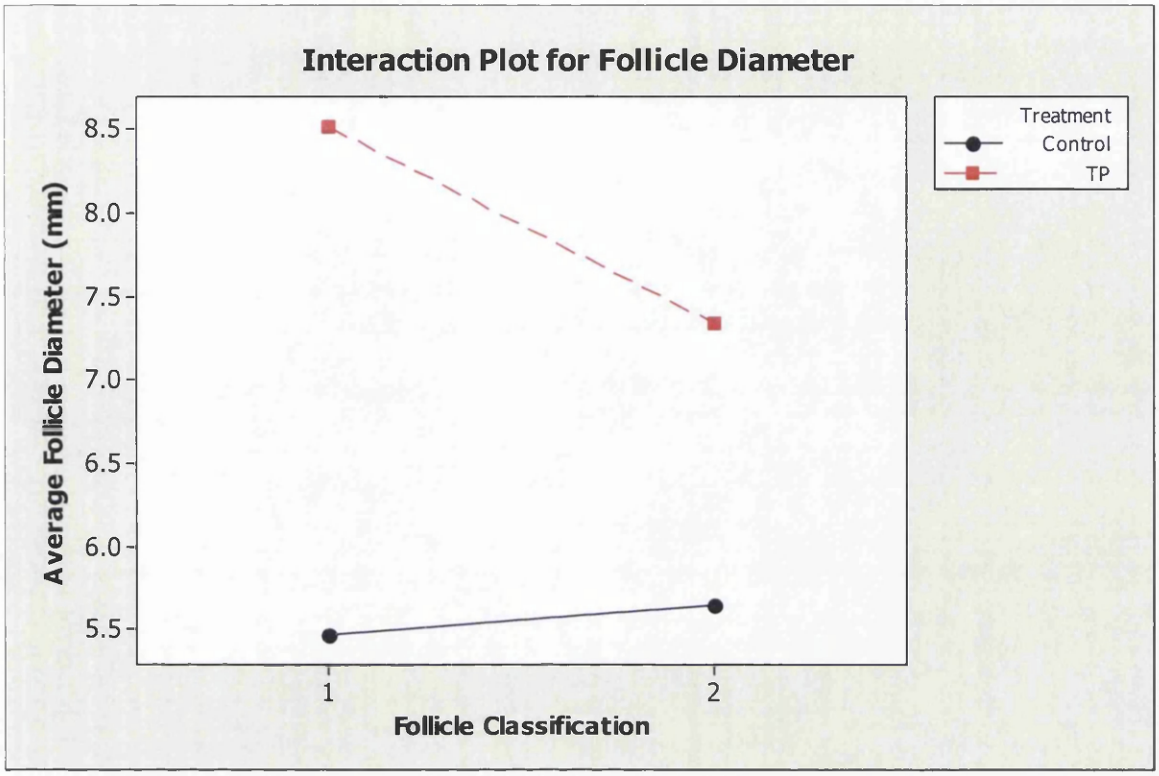


Figure 3.12 Interaction plot for the average follicle diameter recovered from dominant (DF) and subordinate (SF) follicles from control and testosterone propionate (TP) treated ewes. Follicle classification 1 = DF, follicle classification 2 = SF.

3.4 Discussion

The main aim of this study was to determine the effect of prenatal androgen treatment on ovarian function and, specifically, on large antral follicle characteristics (such as follicle diameter and follicular fluid steroid concentrations) in the postpubertal ewe. Results from this study, using five year groups of Dorset sheep, have very much extended earlier findings on the effect of excess TP during foetal life on reproductive dysfunction. Earlier studies examining effects of prenatal TP treatment on various levels of the hypothalamo-pituitary-ovarian axis have used Dorset ewes (Birch *et al.*, 2003; Unsworth *et al.*, 2005), Suffolk and Merino sheep (Clarke *et al.*, 1977; Sharma *et al.*, 2002; Sarma *et al.*, 2005; Steckler *et al.*, 2005; Steckler *et al.*, 2007a), rats (Gorski, 1986) and monkeys (Abbott *et al.*, 2005). In the current study prenatally TP-treated young adult Dorset ewes showed less cyclicity, but increased ovarian weight and elevated peripheral oestradiol concentrations. And for the first time we have now identified intrafollicular abnormalities within the largest follicles present at the beginning of the non-breeding season, which may be responsible for some or all of the systemic steroid changes caused by prenatal exposure to aromatisable testosterone (through TP). Specifically, DF and SF from androgenised ewes showed increased size and intrafollicular oestradiol and progesterone concentrations

compared with controls, but only when animals were androgenised with TP and not DHT. Thus, programmed increased follicular steroidogenesis seen postnatally arises through the oestrogenic action of TP administered to pregnant ewes. The effects of prenatal androgenisation on reproductive cyclicity, peripheral steroid concentrations, ovarian weight and the follicular parameters of the largest two follicles recovered from each ewe are discussed in greater detail below.

At this stage it is pertinent to highlight that significant differences in results (such as ovarian weight and follicular fluid progesterone concentrations) between year groups of ewes approximately the same age were observed. We aimed to investigate the consistent effects of prenatal androgenisation on ovarian function, but interestingly, significant differences were not seen in all years despite significant overall effects using a GLM. These year to year differences may be due to (1) maternal body condition; the breeding ewes in one year group could be of a better condition than in other groups, affecting transit of steroid at the level of the placenta (Jansson *et al.*, 2006; Luther *et al.*, 2007; Jansson *et al.*, 2008) or (2) ewe lamb body condition; the prenatally treated ewes in one year group could gain at a different rate than those in other groups altering responses to programmed changes within the regulatory hormonal axis (Cleal *et al.*, 2007). One further factor must be taken into account. Specifically, one year group stands out from the others in terms of the percentage of control animals having reproductive cycles; year 4, born in 2007. In the late summer of 2006 there was an outbreak of Scrapie at the University Farm and more than half the breeding flock had to be culled. It was difficult to replace these ewes with non pregnant animals at this time of year and eventually a low number of ewe lambs from Northern Ireland were sourced that had been born in January/February 2006. They were bred in November (when about 40 weeks of age) and their lambs were born from late April to the beginning of May 2007. The young age of the mothers and their relatively light weight during gestation, as well as the late time of mating and thus late births may have contributed to the lack of cycles in their offspring in their first breeding season. Ewe lambs may have been too young at the normal time of start of the breeding season, and perhaps had smaller weight gains due to lowered milk yield of the mothers, reducing the number of animals reaching puberty at the start of the breeding season. Further, most of these ewes carried a single pregnancy and the majority had male offspring which is reflected in the very low number of control females that were available for use in the 2007 Year group 4 (n = 3). However, findings from this study over several years still confirm previous results that prenatal TP excess causes not only neuroendocrine abnormalities (Robinson *et al.*, 1999; West *et al.*, 2001; Sharma *et al.*, 2002; Birch *et al.*, 2003; Sarma *et al.*, 2005) but

also altered ovarian function, specifically the occurrence of reproductive cycles and large antral follicle growth and differentiation (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). Thus, this highlights one of the benefits of our experimental approach, i.e. using groups of sheep over several years to determine consistent effects of prenatal androgenisation.

Previous studies using the same breed of sheep clearly demonstrated that significantly fewer TP ewes entered the first breeding season, with similar percentages (64-70%) of TP ewes cycling in the first breeding season to our study (Birch *et al.*, 2003; Unsworth *et al.*, 2005). However, while the studies performed on the Dorset ewes at the Babraham Institute in Cambridge showed that control animals had about 6 progestogenic cycles in the first breeding season (Birch *et a*l, 2003; Unsworth *et al.*, 2005) this has not been the case in studies carried out at the University of Glasgow where animals only had about 3 cycles. The reasons for this probably relate to the late time of mating and thus late time of birth, and the latitude where the animals were maintained. Specifically, lambs were born in Cambridge (52°12'N) in late February meaning that they would have their first reproductive cycle at about 30 weeks of age which would occur in late September/early October. The first breeding season usually ended in mid January some 100 days later which would facilitate the completion of 6 regular cycles. In the case of the lambs born in Glasgow, Scotland (55°52'N) lambs were born later in the year, from late March to late April resulting in the first cycle in November. Cycles normally ceased in early January and so only about 3 regular cycles would be observed.

In the androgenised animals, as was first reported by Clarke, Scaramuzzi and Short (1976, cycles were not regular. The reasons for this are not known, but most likely reflect recognised abnormalities of the positive feedback exerted by oestradiol, control of the formation and lifespan of the *corpus luteum*, and follicular abnormalities. As prenatally TP-treated Dorset ewes are unable to produce a GnRH and thus LH surge due to reduced sensitivity to oestradiol positive feedback at the level of the hypothalamus, (Sharma *et al.*, 2002; Birch *et al.*, 2003; Unsworth *et al.*, 2005), the mechanisms by which prenatally TP-treated ewes can produce some normal looking progesterone profiles indicating reproductive cycles are unknown, given that in a normal ewe the majority of progesterone is secreted by the *corpus luteum* during the oestrous cycle (Berisha & Schams, 2005). One possibility is that luteinisation of follicles in the androgenised ewe may not require a "surge" release of LH, but that a sustained increase in LH pulse frequency observed within the prenatally androgenised ewe (Sarma *et al.*, 2005) is sufficient to luteinise follicular somatic cells of several large differentiated antral follicles. Subsequent timely regression of

the *corpus luteum* or *copora lutea* will depend on the capability of the uterus to produce prostaglandin 2 alpha and release it into the uterine vein (McCracken *et al.*, 1970). As abnormalities in uterine anatomy are present from birth in androgenised ewes (for example, a blind-ending body of uterus not connecting with the vagina), this may lead to abnormal function, i.e. its inability to generate and release enough prostaglandin F2α for luteolysis to occur, which in turn will cause the prolonged progesterone cycles which have been observed.

Elevated peripheral concentrations of oestradiol were identified in three out of the four groups of androgenised ewes studied. We were unable to determine whether differences in peripheral concentrations of oestradiol could be determined from year group 2 ewes in the non-breeding season, as no blood samples were available. To the best of our knowledge, no group has investigated peripheral oestradiol concentrations in the TP-treated Dorset ewe both within and outwith the breeding season. Previous studies in the Suffolk model have determined that the preovulatory oestradiol rise is increased in the prenatal testosterone-but not DHT-treated ewe relative to controls (Veiga-Lopez *et al.*, 2009). Additionally, at 24 weeks of age, circulating concentrations of plasma oestradiol is increased in TP-treated Suffolk ewes (Sarma *et al.*, 2005). Furthermore, during the follicular phase, peak and total peripheral oestradiol produced, as well as total oestradiol released in response to gonadotrophins, are increased in TP-treated Suffolk ewes (Steckler *et al.*, 2008). While the phenotype differs between the two androgenised sheep models, as the ovary intact Suffolk ewe can produce an LH surge in response to oestradiol (Sharma *et al.*, 2002; Veiga-Lopez *et al.*, 2008; Veiga-Lopez *et al.*, 2009) while the ovary-intact Dorset ewe does not (Unsworth *et al.*, 2005 and J E Robinson, unpublished observations), our findings of elevated systemic oestradiol in the Dorset model during the breeding season but also in the non-breeding season very much extend findings from the Suffolk model, which have mostly been determined in normally cycling animals. Thus, both ovulatory (Suffolk model) and non-ovulatory (Dorset model) follicles appear to be secreting abnormally high amounts of oestradiol into circulation of adult prenatally androgenised ewes.

The effects of elevated peripheral oestradiol concentrations could be wide-ranging as oestradiol has many functions both at the neuroendocrine and ovarian levels (Cardenas *et al.*, 2001; Britt *et al.*, 2004). Oestradiol itself has acknowledged local intrafollicular actions (Rosenfeld *et al.*, 2001), stimulating proliferation of granulosa cells in rats (Richards, 1994; Drummond & Findlay, 1999a) and, in synergy with FSH, is responsible for granulosa cell differentiation including aromatase and LH receptor induction (Drummond

et al., 2002). Elevated peripheral concentrations of oestradiol suggests altered ovarian somatic cell steroidogenesis in prenatally TP-treated ewes, with the majority of oestradiol production taking place in the granulosa cells of antral follicles (Hay and Moor 1975). And indeed, the two largest antral follicles from androgenised ewes produced more oestradiol than follicles from control ewes in our study. Oestradiol is also known to reduce granulosa cell apoptosis (Lund *et al.*, 1999; Rosenfeld *et al.*, 2001) and, therefore, increased follicular synthesis of the hormone could explain increased follicle growth leading to the larger diameters observed by us and others, and the persistence observed in prenatally TP-treated ewes where follicles fail to regress (Manikkam *et al.*, 2006). The effects of elevated peripheral oestradiol concentrations at the neuroendocrine level during both breeding season and seasonal anoestrus, however, may be predominantly negative in a model that is characterised by the absence of GnRH/LH surges despite the increased LH pulsatility seen in prenatally TP-treated Suffolk (Sarma *et al.*, 2005) and Dorset (Robinson *et al.*, 1999) ewe. Chronic exposure to excess oestradiol from soon after birth has been shown to delay or prevent the onset of progesterone cycles in females (Foster *et al.*, 1986; Jackson *et al.*, 2009). Evidence of excess oestradiol concentrations affecting the LH surge mechanism is provided by results from chronic post natal exposure to low levels of oestrogen for over a year, starting when lambs were four weeks old, which delayed the induction of the surge mechanism (Malcolm *et al.*, 2006). Exposure of prenatally TP-treated Suffolk ewes to excess oestradiol postnatally (admittedly at a much higher concentration than those detected in our study) resulted in increased progesterone cycle irregularity and abolished the LH surge in more TP-treated ewes compared to those who were not exposed to excess oestradiol postnatally (Jackson *et al.*, 2009). Therefore, the LH surge mechanism in the Suffolk ewe is susceptible to further programming by postnatal exposure to oestradiol, while in the Dorset model the more extreme phenotype may be due to early and continuous systemic elevations in oestradiol.

It must be highlighted that, in the current study, only two blood samples taken three days apart at two time points were used to ascertain peripheral concentrations of oestradiol within and after the end of the breeding season. Therefore, in order to gain a more accurate and perhaps more consistent profile of peripheral oestradiol concentrations within and outwith the breeding season, future investigations should utilise more frequent blood sampling of TP and control ewes over a longer period of time. This infrequent sampling may be one of the reasons why elevated oestradiol was not observed in every year group or in all the December blood samples.

In utero exposure to TP but not DHT increased total ovarian weight in adult ewes, suggesting the pre- and postnatal effects of TP are mediated through the action of oestrogen *in utero*. Previous studies into the effects of prenatal programming by TP and DHT at different stages of development have provided partially conflicting results. In one study, 3 week old Dorset ewes and 5-week old Dorest lambs exposed prenatally to TP demonstrated results similar to this study, determining total ovarian weight to be greater than those of controls (West *et al.*, 2001). However, using the Suffolk breed, ovarian weights were similar to controls in 10 month-old lambs exposed to either TP or DHT (Smith *et al.*, 2008). Interestingly, in Suffolk 140 day old foetuses, ovarian to body weight ratio also appeared to be similar between TP-treated ewes and controls (Steckler *et al.*, 2005), while a more recent study using the same breed of sheep determined that both prenatal TP and DHT treatment increased both ovarian weight and ovarian volume in 140 day old foetuses (Smith *et al.*, 2008). The disparity between results could be due to year effects (see above), but, importantly, also breed differences, as just like the loss of the LH surge, the Dorset sheep could be more sensitive to effects of prenatal TP excess compared to the Suffolk. Thus in the Suffolk phenotype, ovaries recovered from both control and androgenised ewes in the breeding season may contain corpora lutea and large follicles leading to similar weights. Foetal ovaries in the Suffolk phenotype may then show more differences, as earlier activation in TP foetuses will lead to heavier ovaries (Smith *et al.*, 2008). Irrespective of these disparities, increased ovarian weight in the absence of corpora lutea suggests increased ovarian volume, which is related to higher antral follicle numbers in ruminant species (McNatty *et al.*, 1993; Ireland *et al.*, 2008), and thus potentially implies increased follicle survival, or, specifically, increased proliferation or reduced apoptosis of follicular somatic cells. Indeed, this study showed that androgenised ewes had larger DF and SF than control ewes, supporting previous ultrasound-based studies of large antral follicle development in the Suffolk model (Manikkam *et al.*, 2006).

Our approach differs to that of other researchers using the androgenised sheep model in that our research focuses on intrafollicular data acquired from individual DF and SF. Fundamental to our investigation was the correct classification of DF and SF within each ewe. DF and SF classification is generally based on morphological (size) and functional biochemical (oestradiol production) criteria in both the bovine and ovine (Evans & Martin, 2000; Austin *et al.*, 2001; Ryan *et al.*, 2007). Monitoring of ovarian follicle development by transrectal ultrasonography, i.e. tracking follicle growth over a period of several days, is the gold standard to identify dominant and subordinate follicles following selection. Ovarian scanning revealed ovine dominant and largest subordinate follicles reach

maximum diameters of 5-7 and 3-5 mm, respectively (Bartlewski *et al.*, 1999; Evans *et al.*, 2000). DF and SF classification can also be determined by using follicular fluid oestradiol concentrations, the dominant follicle typically characterised by higher follicular fluid concentrations (~41 ng/ml in sheep) than that of the largest subordinate follicle (0.6 ng/ml) in both the ovine (Evans & Martin, 2000) and bovine (Austin *et al.*, 2001). As we were not able to monitor follicle development by transrectal ultrasonography to identify DF and SF following DF selection, our follicle classification was based solely on follicular fluid oestradiol concentrations and largest diameters achieved per ewe. Therefore, it is possible that our SF classification in control ewes does not only include what are traditionally termed the largest subordinate follicle, but also 'co-dominant' follicles, or older dominant follicles, particularly when follicles with less intrafollicular oestradiol were similarly or even larger-sized than the DF. Such follicles are indeed abundant in a sheep breed which on average ovulates 1.8 follicles per cycle (Hall *et al.*, 1986). In androgenised ewes the traditional terms do not appear to apply, as both largest follicles recovered are at least as large as control DF, and at least equally or more steroidogenic than DF in normal ewes (Evans & Martin, 2000). Additionally, based on ultrasound monitoring normal DF selection within androgenised Suffolk ewes does not seem to apply as large follicles remain on ovaries without regression (Manikkam *et al.*, 2006).

Prenatal androgenisation by TP increased follicle diameter of the two largest follicles (both DF and SF) compared to controls. In some androgenised ewes (with TP), more than two follicles reached sizes of greater than 5 mm in diameter, which was never seen in the control ewes. Previous studies in Dorset female lambs aged 3 and 5 weeks have demonstrated that prenatal androgen excess through TP resulted in ovaries containing large antral follicles, in comparison to control ovaries where no follicles greater than 2 mm in diameter were found (West *et al.*, 2001). The same study and subsequent studies determined that the proportion of growing follicles in each size class (primary, secondary, antral) was increased in ovaries from Suffolk and Dorset TP-treated ewes compared with controls (West *et al.*, 2001; Steckler *et al.*, 2005; Smith *et al.*, 2008). Within antral follicles, fluid accumulation is mainly responsible for the increase in diameter, but somatic cells still proliferate (Crisp, 1992; Campbell *et al.*, 1995). Therefore, an increase in follicular fluid volume, or alternatively increased somatic cell proliferation or a combination of both could lead to the increased growth of antral follicles seen in prenatally TP-treated sheep and is most likely the cause of the increased ovarian weight. Continued growth to a larger follicle diameter suggests disruption in the mechanisms controlling

follicle growth and atresia, again perhaps mediated as a result of the intra-ovarian actions of oestrogen as discussed above (Lund *et al.*, 1999).

It is interesting to note that, in our study, no overall difference in follicle diameter between follicles classified as dominant or subordinate was determined. However, an interaction between prenatal treatment and follicle classification was found, as DF with highest intrafollicular oestradiol were also the largest follicle in TP-treated ewes, while this was not always the case in control ewes. Ovarian follicle development in sheep is characterised by 2-4 follicle waves per cycle (Evans *et al.*, 2000), with at least one or more follicles (depending on the breed of sheep) continuing to grow until ovulation. Interestingly, dominance may not be as stringent as in monovulatory species such as the cow as, in some multiple ovulating sheep, follicles from the second last wave may also ovulate (Bartlewski *et al.*, 2000; Driancourt, 2001). In our study of control Dorset ewes usually just one follicle greater than 5.5 mm was present on the ovaries at the end of the breeding season, but did not always have highest intrafollicular oestradiol. Thus our SF classification within each control ewe may have included some old DF (see discussion above), in which oestradiol production begins to decline before morphological regression (Fortune *et al.*, 2001). In comparison in prenatally TP-treated ewes DF and SF were generally larger than 5.5 mm in diameter, and there were often more than 3 follicles with diameters of greater than 4 mm, indicating abnormal large follicle development and a reduced dominance mechanism. Therefore in TP-treated ewes, growth and oestradiol synthesis in large follicles are clearly regulated in parallel, while this is not always synchronous during normal follicle wave development in controls.

Due to the multifollicular appearance of ovaries from young Dorset and Suffolk ewe lambs (West *et al.*, 2001) or postpubertal Suffolk ewes treated with TP *in utero* (Steckler *et al.*, 2005; Manikkam *et al.*, 2006), this prenatally androgenised ewe has been proposed as a model for Polycystic Ovary Syndrome (PCOS), one of the most common female endocrine disorders affecting approximately 5-10% of women of reproductive age (Franks, 1995; Dumesic *et al.*, 2007). Women affected with PCOS present with polycystic ovaries, hypersecretion of LH, in part, from reduced hypothalamic sensitivity to steroid negative feedback, and infertility (Katz & Carr, 1976; Rebar *et al.*, 1976; Norman *et al.*, 2007), all characteristics seen in the prenatally androgenised ewe. There is a debate as to whether the prenatally TP-treated ewe is a relevant model to investigate PCOS in relation to follicle growth and development. The characteristic morphological feature of polycystic ovaries in anovulatory women is accumulation of antral follicles in the range of 2–8 mm in diameter

(Franks *et al.*, 2000). In other words, there is an apparent failure to select a dominant follicle (which always becomes larger than 8 mm), and it is assumed that antral follicles 5 to 8 mm have been arrested in development (Franks *et al.*, 2008). Follicle arrest at the 2 to 8 mm diameter in PCOS women differs from the ovarian phenotype of prenatally TP-treated ewes where, in this investigation and previous studies, antral follicles are regularly observed to be larger than that shown to be DF size, follicles appear to have normal granulosa and theca cell layers and, at least in the Suffolk breed of sheep, are able to respond to an LH surge with ovulation (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a), a marker of the ability to undergo final differentiation. While the cellular changes in such abnormally large follicles have not yet been characterised, it is predicted that the granulosa cells of such highly differentiated and steroidogenic follicles are no longer solely FSH-responsive, a characteristic of PCOS follicles, which also demonstrate reduced granulosa but greatly increased theca cell layers (Franks *et al.*, 2000; Franks *et al.*, 2008). Therefore, there may be two different underlying mechanisms that result in altered terminal follicular development in ovaries from TP-treated ewes versus PCOS women.

Androgenisation by DHT led to intermediate follicle diameters of DF and SF, which did not differ from diameters measured in control or TP-treated ewes. Similar follicle diameters of DF and SF between TP- and DHT-treated groups, while follicle diameters are increased in TP-treated compared to controls ewes, suggest that an increase in follicle diameter is a result of additive effects of both excess androgens and oestrogens. However, previous studies investigating the role of prenatal androgen excess on postpubertal follicular growth determined that abnormalities largely occurred through oestrogenic programming, as the characteristic multifolliculate phenotype was not present in DHT exposed ovaries (Smith *et al.*, 2008). In accordance with the latter study, we never saw examples of multiple large follicles on ovaries from DHT-treated ewes in year group 5, despite the lack of significant size differences between the largest follicles recovered from DHT- and TP-treated ovaries. Clearly, this requires further examination over several years.

Prenatal androgenisation by TP increased follicular fluid oestradiol concentration in both DF and SF compared to follicles recovered from control and DHT-treated animals. Androgenisation by DHT had no effect on follicular fluid oestradiol concentration compared to controls. Therefore, increased follicular oestradiol synthesis by large antral follicles of adult TP-treated ewes arises as a result of oestrogenic programming *in utero*. Follicular fluid oestradiol concentrations within DF recovered from control ewes (~35 ng/ml) were similar to those detailed in previous studies (Murdoch & Dunn, 1982;

Jolly *et al.*, 1997; Evans & Martin, 2000). Oestradiol plays an important role in reproduction and other biological processes regulating the growth, differentiation and function of reproductive tissues, as well as of a number of other organs, such as bones, the brain and the cardiovascular system (Drummond, 2006). Oestradiol is required for follicle development past the antral stage as determined in CYP19A1 knockout mice (Britt *et al.*, 2001). Additionally, CYP19A1 knockout mice have reduced numbers of primordial and primary follicles and this reduction is not corrected by postnatal oestradiol treatment, suggesting that oestradiol stimulates the initial formation or activation of primordial follicles (Britt *et al.*, 2004). Thus, both these results demonstrate that oestradiol has a role in initial and terminal follicle development. Therefore, increased concentrations of oestradiol *in utero* as a result of aromatisation of excess testosterone could lead to the increased recruitment seen in Suffolk androgenised ewes through the action of oestrogenic programming (Steckler *et al.*, 2005; Smith *et al.*, 2008). Postnatally, this increase in recruitment may be maintained as a result of increased oestradiol synthesis within large antral follicles leading to higher than normal intra-ovarian oestradiol, and the consequence will be accelerated depletion of the primordial follicle pool.

Follicular fluid oestradiol concentrations (and the oestrogen: progesterone ratio) are directly related to follicle health and granulosa cell differentiation (Evans & Martin 2000). Oestradiol synthesis is mediated by the aromatase enzyme in granulosa cells and higher availability of androgen precursors from the theca (Rosenfeld *et al.*, 2001). The fact that testosterone levels were not increased in follicular fluid of follicles from TP-treated ewes may point to a very functional aromatase system, able to convert extra precursor into oestradiol. Increased production and secretion of oestradiol into the circulation from multiple large follicles may, thus, be responsible for increased plasma oestradiol concentrations detected in prenatally TP-treated ewes in our study for both the breeding and the non-breeding season. Oestradiol production by granulosa cells is dependent on FSH during cohort emergence, but on LH following selection (Campbell *et al.*, 1995), and the increased LH pulsatility determined previously in prenatally TP-treated ewes (Sarma *et al.*, 2005) may, thus, be responsible for maintaining high oestradiol synthesis in the large antral follicles. However, while the principle of increased follicular oestradiol production being due to increased LH pulsatility clearly applies to DF growth and persistent DF in normal ruminants (Roberson *et al.*, 1989; Kojima *et al.*, 1992), it may not be the case in androgenised ewes treated with TP, as excess DHT *in utero* also leads to increased LH pulsatility but not to enhanced large antral follicle oestradiol production (Veiga-Lopez *et al.*, 2009). It is possible that higher differentiation in larger antral follicles of TP

androgenised ewes is accompanied by an increase in LH responsiveness, for example due to higher receptor expression similar to what is seen in growing DF in cattle (Mihm et al 2006), which allows such follicles to respond maximally to the elevated LH. While such investigations are the subject of the next chapters, increased (inherent or possibly LH-stimulated) follicular oestradiol production may then have the consequence of continued somatic cell survival and proliferation leading to the abnormally large sizes determined for the follicles recovered from TP-treated ewes in this study.

Prenatal androgenisation by TP increased follicular fluid progesterone concentration in both DF and SF from adult ewes compared to follicles recovered from control and DHT-treated animals. Androgenisation by DHT had no effect on follicular fluid progesterone concentration compared to controls. Therefore, increased follicular fluid progesterone concentration in large antral follicles of prenatally TP-treated ewes arises as a result of oestrogenic programming. As far as we are aware, no previous literature exists on follicular fluid progesterone concentrations from individual large antral follicles recovered from prenatally androgenised ewes. Elevated progesterone concentrations in the follicular fluid of follicles recovered from prenatally TP-treated ewes may imply that the follicle is luteinising prematurely in the absence of a LH surge and ovulation. Thus, granulosa and theca cells may be differentiating prematurely into large and small luteal cells as occurs normally after the surge but before the expulsion of the oocyte (Fortune *et al.*, 1994) possibly due to the elevated LH pulsatility present in prenatally TP-treated ewes (Sarma *et al.*, 2005). However, there clearly is no concomitant fall in oestradiol normally seen in preovulatory follicles after the surge (Murdoch & Dunn, 1982), as the oestradiol to progesterone ratio in DF and SF is similar in control and TP-treated ewes. Additionally, granulosa cell morphology visualised during harvest (see Chapter 6) is unaltered. Thus, these two findings suggest that in TP-treated ewes follicular somatic cells are prevented from luteinisation despite abnormally high progesterone synthesis. Similarly, dominant follicles following their loss of dominance and dominant follicles after prolonged persistence due to maintained frequent LH pulses in the bovine also increase their progesterone synthesis, as can older cystic follicles (Calder *et al.*, 2001; Bridges & Fortune, 2003; Vanholder *et al.*, 2006). In all these cases, however, oestradiol synthesis is reduced and, thus, the oestrogen to progesterone ratio is lowered, while in our study, unusually, both progesterone and oestradiol synthesis appear similarly increased in follicles from TP-treated ewes.

Similar to oestradiol, progesterone is required for many aspects of female reproductive function including sexual behaviour, mammary gland development, ovulation, implantation and the maintenance of pregnancy (Brosens *et al.*, 2004; Peluso, 2006). Intra-ovarian actions of progesterone include inhibition of granulosa cell proliferation independent of its ability to influence gonadotrophin levels (Hirshfield, 1984). Additionally, progesterone itself has been shown to enhance granulosa cell progesterone secretion in cultured rat granulosa cells (Schreiber *et al.*, 1980), and slows the rate of mitogen-induced proliferation of granulosa cells in rats and humans (Chaffkin *et al.*, 1993; Peluso & Pappalardo, 1998). It is difficult to predict the intra-ovarian role progesterone has on large antral follicles. Interestingly, progesterone prevents apoptosis of rat granulosa cells (Peluso & Pappalardo, 1998; Svensson *et al.*, 2000; Shao *et al.*, 2003), and such increased cell survival could further explain the persistent large antral follicles observed on TP-treated ovaries. In addition, we speculate that enhanced intrafollicular oestradiol counteracts the anti-proliferative effects of progesterone leading to continued growth of large antral follicles in TP-treated ewes.

Prenatal androgenisation by TP had no effect on follicular fluid testosterone concentrations. However, SF had higher intrafollicular testosterone than DF. This may be explained by lower aromatisation of androgen precursor produced by theca cells in SF, and thus accumulation of androgen in follicular fluid, similar to what has been described for atretic follicles in sheep (Tsonis *et al.*, 1984).

In conclusion, results in this study have clearly demonstrated for the first time that abnormal high steroidogenesis within DF and SF in androgenised ewes is programmed prenatally through oestrogenic action of TP. It remains to be seen, however, which cellular factors (genes) or pathways mediate the changes in granulosa and/or theca cell steroidogenesis and proliferation/apoptosis proposed for the highly steroidogenic and abnormally large follicles recovered from TP-treated ewes.

CHAPTER 4. EFFECT OF PRENATAL TESTOSTERONE TREATMENT ON STEROIDOGENIC ENZYME EXPRESSION AND ATRESIA IN SMALL ANTRAL FOLLICLES OF ADULT EWES

4.1 INTRODUCTION

The multifollicular ovarian morphology first observed in prenatally TP-treated Dorset and Suffolk ewes (West *et al* 2001), and recently studied in more detail (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a), may be the result of increased follicular recruitment and/or, alternatively, of follicular persistence from a failure to regress (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). Ovine large antral follicles are described as persistent if they are observed on the ovary for 12 or more days (Flynn *et al.*, 2000). Follicular persistence as a result of prenatal TP has been demonstrated in the Suffolk ewe, leading to increasing sizes and longer lifespan of the largest follicles during both the first and second breeding seasons (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). Additionally, when antral follicles from 3mm were grouped into three size classes that functionally correspond to FSH-dependent cohort follicles (3-4 mm), follicles selected for ovulation (4-8 mm), and follicles larger than ovulatory size follicles (8 mm) (Campbell *et al.*, 1995; Driancourt, 2001), follicles within each size class stayed longer in the ovary of TP exposed ewes compared to control follicles (Manikkam *et al.*, 2006). Furthermore, this follicular persistence is programmed by oestrogenic and not androgenic action of prenatal TP, as TP, but not DHT treatment, increased the number of large follicles (Steckler *et al.*, 2007a).

Chapter 3 demonstrated that prenatal androgenisation by TP results in enhanced growth within DF and SF leading to an enhanced ability to synthesize oestradiol and progesterone. It is not known at what stage of follicle development prenatal androgenisation by TP enhances follicle differentiation. Previous studies investigating the effect of prenatal exposure to excess testosterone on early follicle development (follicles less than 1 mm in diameter) in foetal ovaries (day 90 and 140 of gestation) and in adult ovaries (10 months of age) showed enhanced follicle recruitment (Steckler *et al.*, 2005; Forsdike *et al.*, 2007; Smith *et al.*, 2008), increased numbers of antral follicles (Forsdike *et al.*, 2007; Steckler *et al.*, 2007a) and increased oestrogen receptor alpha and androgen receptor protein in granulosa cells of antral follicles (Ortega *et al.*, 2009). Thus, more recruitment appears to also lead to more follicles developing to the antral stages, and antral follicles may be able to respond more to intra-ovarian oestradiol and androgen. However, to the best of our knowledge no group has characterised in detail follicle health, thus continued survival, and onset of gonadotrophin-dependent follicle differentiation, i.e. the ability to synthesize steroids, following antrum formation in androgenised ewes. The enhanced ability of early antral follicles to be able to synthesize and respond to oestradiol and other steroids, and

thus avoid atresia and continue development in higher than normal numbers (due, for example, to the antiapoptotic and proliferative effects of oestradiol), may be significant in the development of the multifollicular phenotype described for the androgenised ewe.

The studies described in this chapter had three main aims. The primary objective was to determine whether the follicular morphology observed in prenatally TP-treated ewes is as a result of a reduction in antral follicle atresia. The second aim was to determine whether the size distribution within antral stages is altered as a result of excess TP *in utero,* possibly indicating abnormal follicle development. The final goal was to determine the steroidogenic capability and differentiation status, specifically the presence of CYP19A1 and HSD3B1 proteins within granulosa and theca cells, of antral follicles ranging from 0.2-4mm in diameter from control and prenatally TP-treated ewes, thus spanning gonadotrophin-independent (0.2 to 3 mm) and –dependent (>3 mm; specifically FSH-dependent) follicle stages (Dufour *et al.*, 1979; Campbell *et al.*, 1995). In these studies it was important to use good markers of follicular differentiation and the two enzymes fulfil this role. Specifically, aromatase (CYP19A1) and 3 beta-hydroxsteroid dehydrogenase (HSD3B1) are responsible for the production of oestradiol and progesterone, respectively, in follicular granulosa and theca cells (Logan *et al.*, 2002; Amsterdam *et al.*, 2003). Both these steroid enzymes are markers of gonadotrophin-dependent follicle differentiation: CYP19A1 is present solely in granulosa cells from follicles greater than 3.5 mm in diameter and its expression deceases in atretic follicles (Huet *et al.*, 1997), while HSD3B1 is initially only localised to theca cells (Logan *et al.*, 2002), but is usually present within granulosa cells of preovulatory follicles (Conley *et al.*, 1995; Webb *et al.*, 1999a) in addition to primary and secondary follicles (Conley *et al.*, 1995; Logan *et al.*, 2002). Thus, HSD3B1 is expressed in granulosa cells of early preantral follicles but then is absent until the preovulatory stage. Therefore, expression of both enzymes has been shown to be upregulated in ruminant granulosa cells from highly differentiated, selected follicles (Bao & Garverick, 1998).

In order to achieve these objectives, ovaries were harvested from control and TP-treated ewes at 10- (year group 3) and 22-months of age (year group 1) to investigate the effect of prenatal androgen treatment on small antral follicle health and steroidogenesis. Follicle health was investigated by histological analysis of atresia using haematoxylin and eosin staining, and by immunofluorescence to determine the presence or absence of the pro-apoptotic Caspase 3 (CASP3) protein in follicular somatic cells. Studies of various experimental models of apoptosis suggested that the presence of active CASP3 is a reliable

indicator of apoptosis (Gown & Willingham, 2002; Duan *et al.*, 2003; Yacobi *et al.*, 2004). Immunofluorescence was also utilised to determine the presence or absence of CYP19A1 and HSD3B1 proteins in follicular somatic cells from all individually identified antral follicles 0.2-4 mm in diameter.

4.2 Materials and Methods

4.2.1 Animals and Prenatal Treatment

Poll Dorset ewes were androgenised *in utero* using an established model (Robinson *et al.*, 1999; Steckler *et al.*, 2005) and euthanased at 10- (Control = 7, TP = 10) and 22-months of age (Control = 9, TP = 7 ewes) for ovary collection.

4.2.2 Quarter Ovary Collection

Ovaries were collected in February when most ewes (94%) were out of the breeding season. Following excision of the largest antral follicles (controls from 3.5 mm, TP from 5 mm) for subsequent follicular studies presented in chapter 3 the remaining ovarian tissue was quartered, and complete quarters were then either snap-frozen in liquid nitrogen (22 months old animals) or wrapped up in tinfoil then placed on dry ice (10 month old ewes) and then stored at -80°C for subsequent histology and immunofluorescence.

4.2.3 Histology

Sections were cut as described in section 2.10. Serial sections were cut at a thickness of 14 μm and each quarter ovary produced between 100 and 270 sections. Following sectioning, all slides were stored at -80°C until processing. A proportion of the frozen ovarian sections were histologically stained to allow for the identification of follicular structures. Frozen ovarian sections were quickly thawed and fixed for 20 minutes in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer. Following fixation, sections were then washed and histologically stained with haematoxylin and eosin as described in section 2.10, coated in DPX mountant and finally cover-slips were attached.

4.2.4 Antral Follicle Identification and Size Measurements

Haematoxylin and eosin staining of every 10th section was used to determine the number and sizes of antral follicles, and antral follicle health in quarter ovaries from TP and control sheep. Images of each sequential section were captured under a light microscope (Leica DM4000, Leica Microsystems (UK) Ltd., Milton Keynes, UK). Ovarian maps were drawn from sequentially captured images to enable individual identification of all antral follicles and ensure no duplication of follicles analysed. Antrum formation begins when follicles

reach 0.15-0.2 mm in diameter (Campbell *et al.*, 1995; Webb *et al.*, 1999b), and thus all antral follicles from 0.2 mm in diameter were counted and their diameter determined from the section which showed individual follicles at their largest size. The mean diameter was determined by taking two measurements at right angles to each other from and to the basal lamina (Irving-Rodgers *et al.*, 2001) using the QWin program (Leica Microsystems (UK) Ltd., Milton Keynes, UK). Follicles were classified into three size classes, specifically ≥0.2-<1 mm (early antral, gonadotrophin-independent), 1-2 mm (acquisition of FSH dependence) and >2 mm (FSH dependent) (Dufour *et al.*, 1979; Miller *et al.*, 1979; McNeilly, 1984; Campbell *et al.*, 1995). As stated above, the largest follicles had already been excised from the control and TP ovaries.

4.2.5 Assessment of Follicle Health

Deciding when a follicle has entered atresia remains somewhat subjective and various criteria have been used to determine the health of antral follicles histologically (Irving-Rodgers *et al.*, 2001; Rodgers & Irving-Rodgers, 2009). All antral follicles individually identified were classified as healthy or atretic (Irving-Rodgers *et al.*, 2001; Rodgers & Irving-Rodgers, 2009). Follicle health classification in this study was based on the number of pyknotic granulosa cells, separation of the granulosa layer from the basal lamina, and disruption of the theca layer. Pyknotic bodies represent a relatively late stage in apoptosis, and have been widely used in the histological classification of atretic sheep and cattle ovarian follicles (Marion *et al.*, 1968; Cahill *et al.*, 1979; Driancourt *et al.*, 1985; Driancourt, 1987). In this study we defined healthy follicles as having no/few minor degenerative changes with ≤2 pyknotic bodies per field of view (Driancourt & Mariana, 1982). Atretic follicles were characterised by a destruction of follicular structure (large parts of the granulosa cell layer separated from the basement membrane, a disrupted basement membrane, apoptotic granulosa cells (more than 2 pyknotic bodies per field of view), and/or a noticeable decrease in the number of granulosa cells within the granulosa cell layer (Driancourt & Mariana, 1982). Representative images of the follicular morphology of follicles classified as either healthy or atretic are shown in Figures 4.1 and 4.2. To confirm follicle health status, as determined by the aforementioned histological markers, immunofluorescence for the presence of the activated form of the pro-apoptotic marker CASP 3 was performed (Gown & Willingham, 2002; Duan *et al.*, 2003; Yacobi *et al.*, 2004).

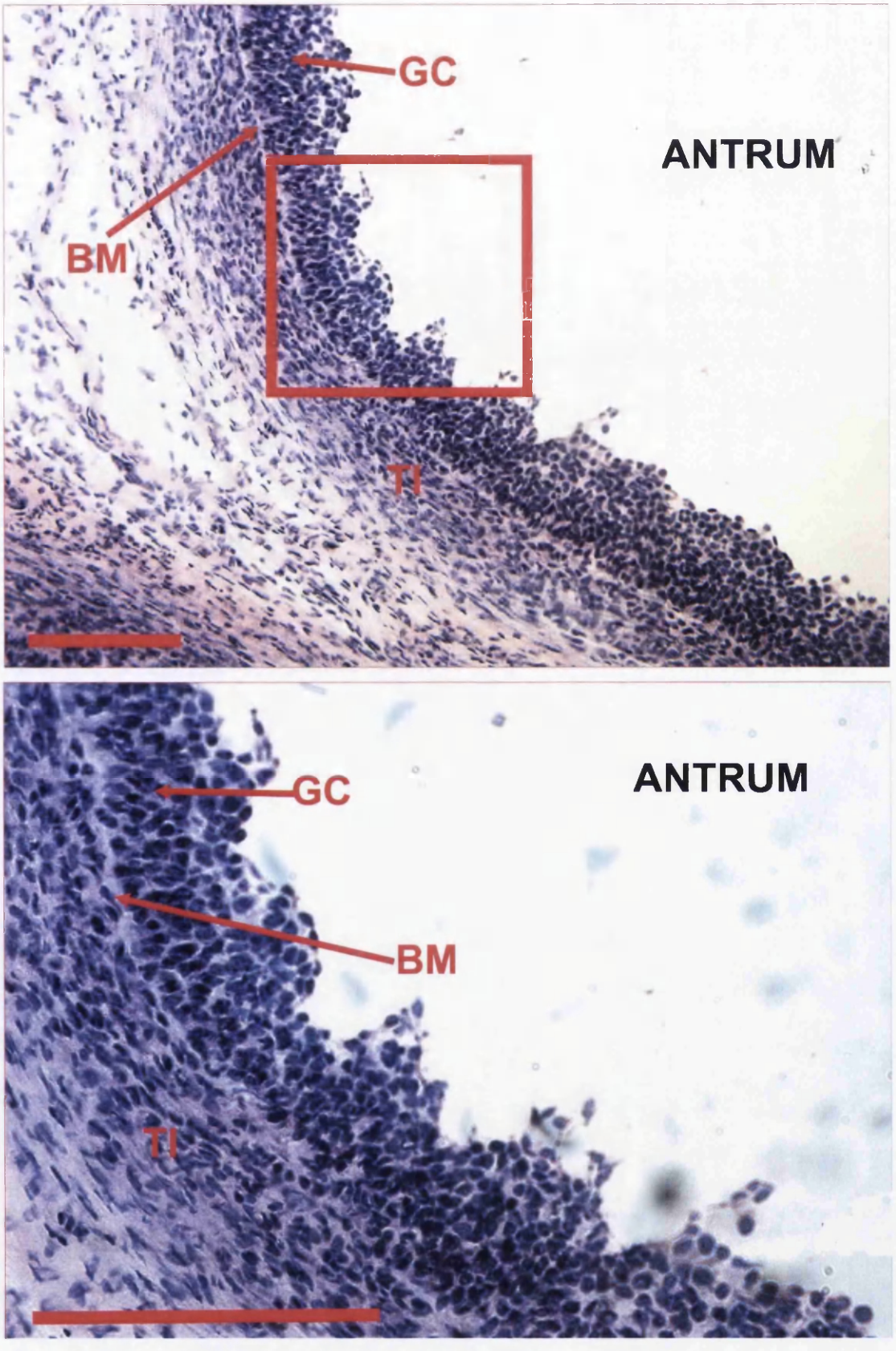


Figure 4.1 Representative images of follicles classified as healthy based on morphological features. Follicles classified as healthy had little or no pyknosis of granulosa cells and granulosa cells were ordered and closely packed to the basement membrane with little or no separation between cells. GC = granulosa cells; BM = basement membrane; TI = theca interna. Scale bars = 100 μm

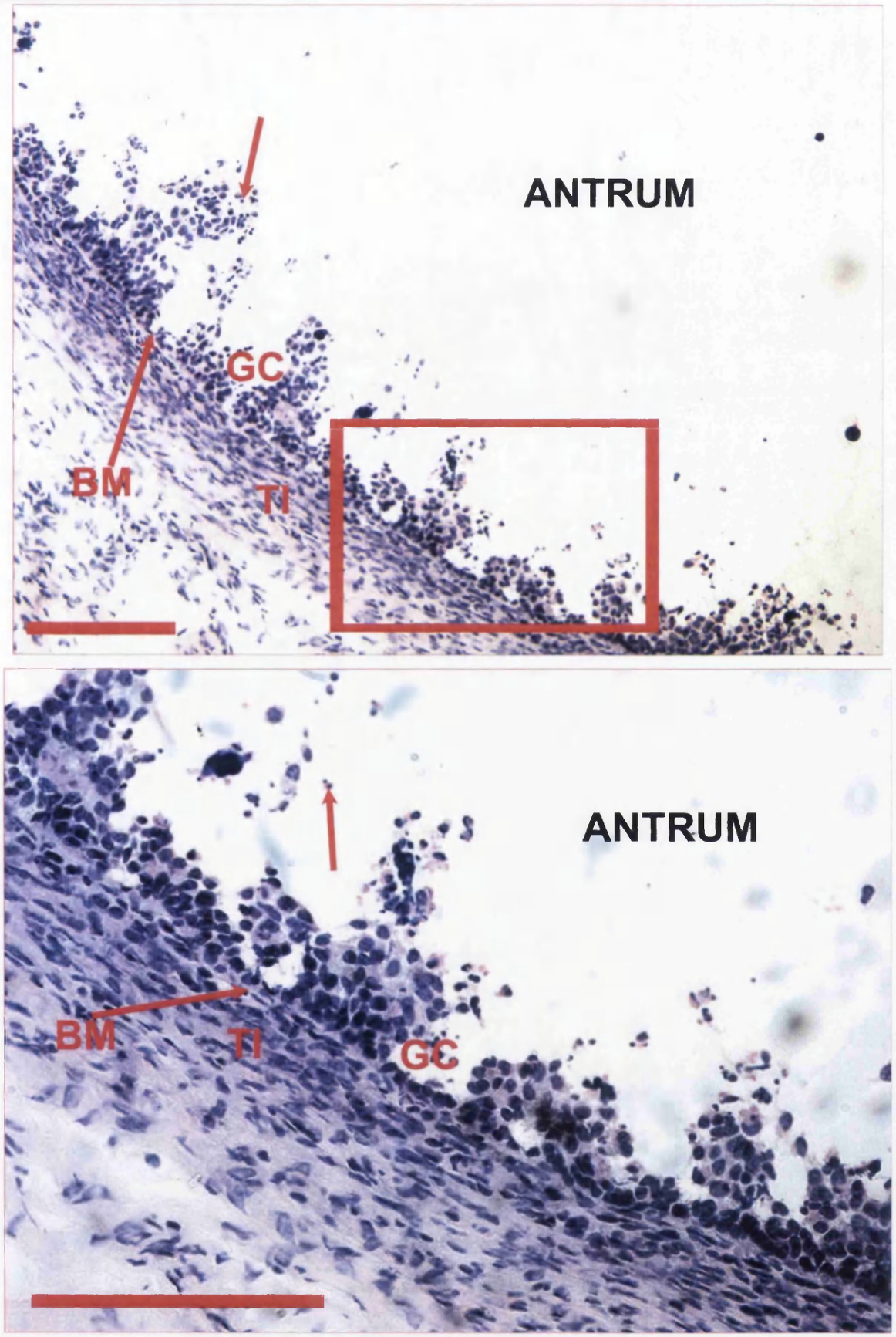


Figure 4.2 Representative images of follicles classified as atretic based on morphological features. Follicles classified as atretic displayed pyknosis in several granulosa cells and the granulosa cells were separated from the basement membrane. GC = granulosa cells; BM = basement membrane; TI = theca interna. Scale bars = 100 μm

4.2.6 Immunofluorescence

The immunofluorescence protocol used was as described previously (Bellingham *et al.*, 2009), without the need for antigen retrieval prior to primary antibody incubation. Primary antibodies used in the study were: (1) CASP3 (rabbit polyclonal against activated form of human/mouse CASP3; AF835, R&D Systems, Minneapolis, MIN, USA), (2) HSD3B1 (rabbit polyclonal antibody against human recombinant type I HSD3B; kindly supplied by Dr. J.I. Mason, University of Edinburgh; validated for sheep gonads (Quirke *et al.*, 2001) and (3) CYP19A1 (mouse monoclonal antibody against human CYP19A1; MCA1974T, AbD Serotec, Oxford, UK).

4.2.7 Statistical Analyses

A Students t-test was used to determine any differences in the total number of follicles analysed per ewe between prenatal treatment groups. A Fisher's exact test was used to determine any differences in the proportion of follicles classified as healthy within each age group and each of age group of the same prenatal treatment. A Fisher's exact test was also used to determine any differences in the proportion of all antral follicles, the proportion of all follicles (and healthy follicles only) within each size class (0.2-1 mm, >1-2 mm and >2 mm) between prenatal treatment and age groups.

A Fisher's exact test was used to determine differences in the proportion of follicles immunoreactive for HSD3B1 in granulosa and theca cells and CYP19A1 in granulosa cells over all sizes class and within each size class.

4.3 Results

4.3.1 Antral Follicle Numbers and Health

The average total number of antral follicles per ewe was increased ($P < 0.05$) by prenatal androgenisation when ewes were 10 months of age, however by 22 months of age this increase was lost (Table 4.1). A higher proportion of antral follicles were histologically classified as healthy (36.9%) in quarter ovaries from TP-treated ewes than in control ovaries (19%) when ewes were 10 months old ($P < 0.001$). No difference in the proportion of healthy antral follicles between the two treatment groups was found in the quarter ovaries from ewes aged 22 months. A higher proportion of healthy antral follicles were present in control ewes at 22 months of age compared to when ewes were only 10-months old ($P < 0.05$). Immunofluorescence for the active form of CASP3, the pro-apoptotic marker, showed that CASP3 was found in granulosa, theca and interstitial cells (Figure 4.3). Thirty-nine and seventy-seven antral follicles from 0.2 mm in diameter were analysed

for activated CASP3 expression from quarter ovaries recovered from control and prenatally TP-treated ewes in year group 5, respectively. Of the analysed follicles, none of those classified as healthy by histological features in either treatment group contained any granulosa or theca cell in which active CASP3 was present. This confirmed that follicles were indeed healthy as histological and immunofluorescent evaluation of follicle health concurred (Table 4.2). The majority of follicles classified as atretic by histology (26/30 for control and 40/52 for TP ovaries) were found to have >20 granulosa or theca cells positive for active CASP3, thus histological and immunofluorescent evaluation of follicle atresia concurred.

	10 months of age		**22 months of age**	
	Control (n=7)	TP (n=10)	Control (n=9)	TP (n=7)
Total number of antral follicles analysed per treatment group	105[a]	298[b]	183[a]	179[a]
Total number of antral follicles analysed per treatment group/ewe	15[a]	30[b]	20[a]	~25[a]
Percentage of antral follicles classified as healthy	19.0%[a]	36.9%[b]	33.9%[a]	29.6%[a]

Table 4.1 Effect of prenatal testosterone on the proportion of healthy antral follicles from 0.2 mm in diameter in ewes aged 10 and 22 months. Follicle health was determined histologically by haematoxylin and eosin staining [a,b] Within a year group, percentages without a common superscript differ ($P < 0.05$).

4.3.2 Antral Follicle Size

There was no difference between treatments in the proportion of follicles (healthy and atretic) belonging to each size class (Table 4.3). Clearly, the overall majority (80-90%) of antral follicles individually identified in quarter ovaries from experimental sheep was less than 1 mm in diameter, with approximately two third classified as atretic (Tables 4.3 and 4.4). At both 10- and 22-months of age there was no difference between the two treatment groups in the proportion of healthy antral follicles classified into the two size classes larger than 1mm (>1-2 mm, >2 mm) (Table 4.4). However, at 10 months of age androgenisation by TP increased the proportion of healthy follicles in antral follicles 0.2-1 mm in diameter. The proportion of healthy follicles in the >1-2 mm and >2 mm size classes increased with age ($P < 0.05$), and in control ewes the proportion of follicles in the larger size classes (>1-2, and >2 mm) also increased with age ($P < 0.05$).

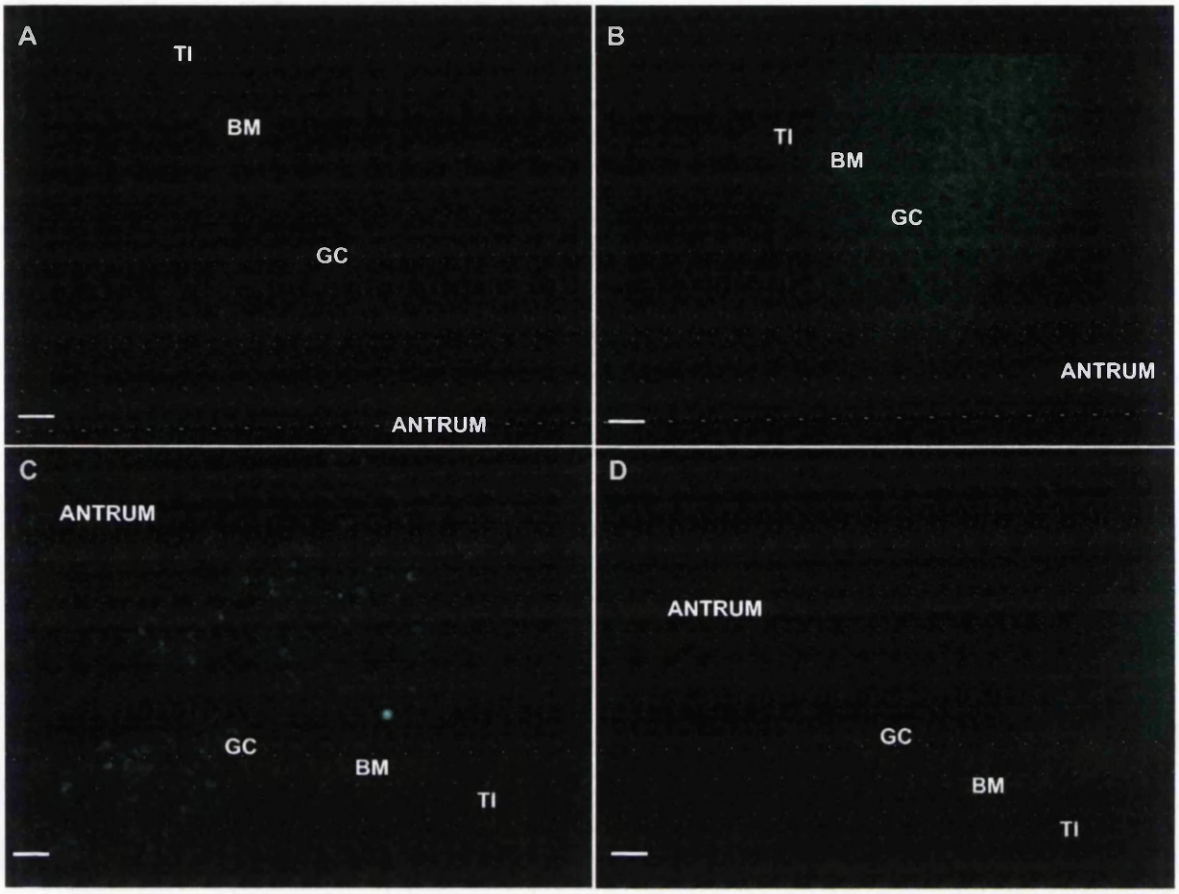


Figure 4.3 Representative images of active caspase 3 (CASP3) expression in ovine antral follicles. (**A**) CASP3 is not present in granulosa and theca cells in follicles classified as healthy. (**B**) Corresponding negative control for follicle in panel (A). (**C**) CASP3 is localized to granulosa cells (labelled) of an antral follicle classified as atretic. (**D**) Corresponding negative control for follicle in panel (C). GC = granulosa cells; BM = basement membrane. TI = theca interna. Scale bars = 20μm.

	Control	TP
Total number of antral follicles analysed	39	77
Percentage of antral follicles classified as healthy but with at least one granulosa or theca cell positive for CASP3 staining	0% (0/9 follicles)	0% (0/25 follicles)
Percentage of antral follicles classified as atretic with +ve CASP3 staining out of all follicles histologically classified as atretic	86.7% (26/30 follicles)	76.9% (40/52 follicles)

Table 4.2 Summary of the immunohistochemistry study investigating activated caspase 3 (CASP3) immunoreactivity in granulosa and theca cells of healthy and atretic follicles within quarter ovary sections obtained from a subset of animals from the 22 month old group. C = 2; TP = 2 ewes.

	10 months old		22 months old	
Follicle Diameter	**Control (n=7)**	**TP (n=10)**	**Control (n=9)**	**TP (n=7)**
>2 mm	3.81%	3.69%	6.56%	3.91%
>1-2 mm	6.67%	9.75%	13.11%	13.41%
0.2-1 mm	89.52%	86.56%	80.33%	82.68%

Table 4.3 Percentage size distribution of all antral follicles (healthy and atretic) into three follicle diameter classes (0.2-1 mm, between 1-2 mm and >2 mm) in prenatally testosterone propionate (TP) treated and control sheep when 10- or 22-months old.

	10 months old		22 months old	
Follicle Diameter	**Control (n=7)**	**TP (n=10)**	**Control (n=9)**	**TP (n=7)**
>2 mm	0.95%	1.68%	2.73%	1.68%
>1-2 mm	0.95%	3.71%	6.01%	6.14%
0.2-1 mm	17.20%[a]	31.52%[b]	25.14%	21.79%

Table 4.4 Percentage of antral follicles classified as healthy by morphology into three follicle diameter classes (0.2-1 mm, between 1-2 mm and >2 mm) in prenatally testosterone propionate (TP) treated and control sheep when 10- or 22-months old. [a,b] Within a year group, percentages without a common superscript differ ($P < 0.05$).

4.3.3 Antral Follicle Steroidogenesis

A summary of the immunocytochemical results relating to the expression of the steroidogenic enzymes HSD3B1 and CYP19A1 in quarter ovaries from ewes aged 10 and 22 months is shown in Table 4.5. A representative immunolocalization image of HSD3B1 and CYP19A1 in ovine antral follicles positive for each enzyme is shown in Figure 4.4. The enzyme HSD3B1 was detected in theca, granulosa and luteal cells, while CYP19A1 was present solely in the granulosa cells, as expected.

Ten Month Old Lambs (Table 4.5)

Approximately three times more small antral follicles were found (and analysed) in the TP compared with the control quarter ovaries, as was noted earlier in this chapter. Of these, a substantial proportion exhibited immunoreactivity for HSD3B1 in thecal cells and this proportion did not differ between the control and androgenised animals (C, 65.00%; TP, 70.59%). A much lower percentage of follicles contained granulosa cells that were immunoreactive for this enzyme and, although this value was twice as large in the control animals, it was not significantly different (C, 7.27%; TP, 13.73%). Granulosa cells from four antral follicles less than 1 mm in diameter were immunoreactive for HSD3B1, and this was only noted in ovaries from TP-treated animals.

CYP19A1 was confined solely to granulosa cells, and we did not observe any follicles containing this enzyme in thecal cells. About twice the proportion of follicles in TP

animals contained immunoreactive granulosa cells compared to the controls (TP, 18.30%; C, 9.09%), although this proportion did not differ significantly. The majority of immunoreactive follicles were larger than 1 mm in diameter with only 1.8% (C) and 4.6% (TP) of the <1mm antral follicles labelled for CYP19A1.

Twenty-Two Month Old Ewes (Table 4.5)

Between one third and one half of all antral follicles in quarter ovaries from the older animals had thecal cells immunoreactive for HSD3B1 and this was not different between the control and androgenised animals (C, 43.76%; TP 37.93%). In marked contrast, only one antral follicle (1/183) from the combined treatment groups showed granulosa cell immunoreactivity for HSD3B1, and this follicle belonged to the control ovaries. Relatively few antral follicles showed granulosa cell immunoreactivity for CYP19A1 (C, 17.71% diameter range 0.6-2.88 mm: TP, 25.29%, diameter range 0.42-2.36 mm) and this did not differ between treatment groups. A higher ($P<0.05$) proportion of follicles with granulosa cell immunoreactivity for CYP19A1 was detected in the very small (≤1 mm) antral follicle size class (C, 1.04%; TP, 11.49%) from prenatally TP-treated ovaries ($P < 0.05$).

Immunohistochemical Analysis	10 Months of age		22 Months of age	
	Control (n=7)	TP (n=10)	Control (n=9)	TP (n=7)
Total number of antral follicles analyzed for ICC	55[a]	153[b]	96[a]	87[a]
HSD3B1 in TH cells from all sizes of analyzed follicles	36 (65.00%)[a]	108 (70.59%)[a]	42 (43.76%)[a]	33 (37.93%)[a]
HSD3B1 in GC cells from all sizes of analyzed follicles	4 (7.27%)[a]	21 (13.73%)[a]	1 (1.04%)[a]	0 (0%)[a]
HSD3B1 in GC cells from follicles <1 mm in diameter	0 (0%)[a]	4 (2.61%)[a]	0 (0%)[a]	0 (0%)[a]
CYP19A1 in GC from all sizes of follicles analyzed	5 (9.09%)[a]	28 (18.30%)[a]	17 (17.71%)[a]	22 (25.29%)[a]
CYP19A1 in GC from follicles <1 mm in diameter	1 (1.82%)[a]	7 (4.58%)[a]	1 (1.04%)[a]	10 (11.49%)[b]

Table 4.5 Number and percentage (brackets) of small antral follicles immunoreactive for the steroidogenic enzymes HSD3B1 and CYP19A1 in control and prenatally androgenised (TP) ewes aged 10 and 22 months. n = number of animals per group.[a,b] Within an age group, percentages without a common superscript differ ($P < 0.05$). IHC = immunocytochemistry, GC = granulosa cells, TH = theca cells.

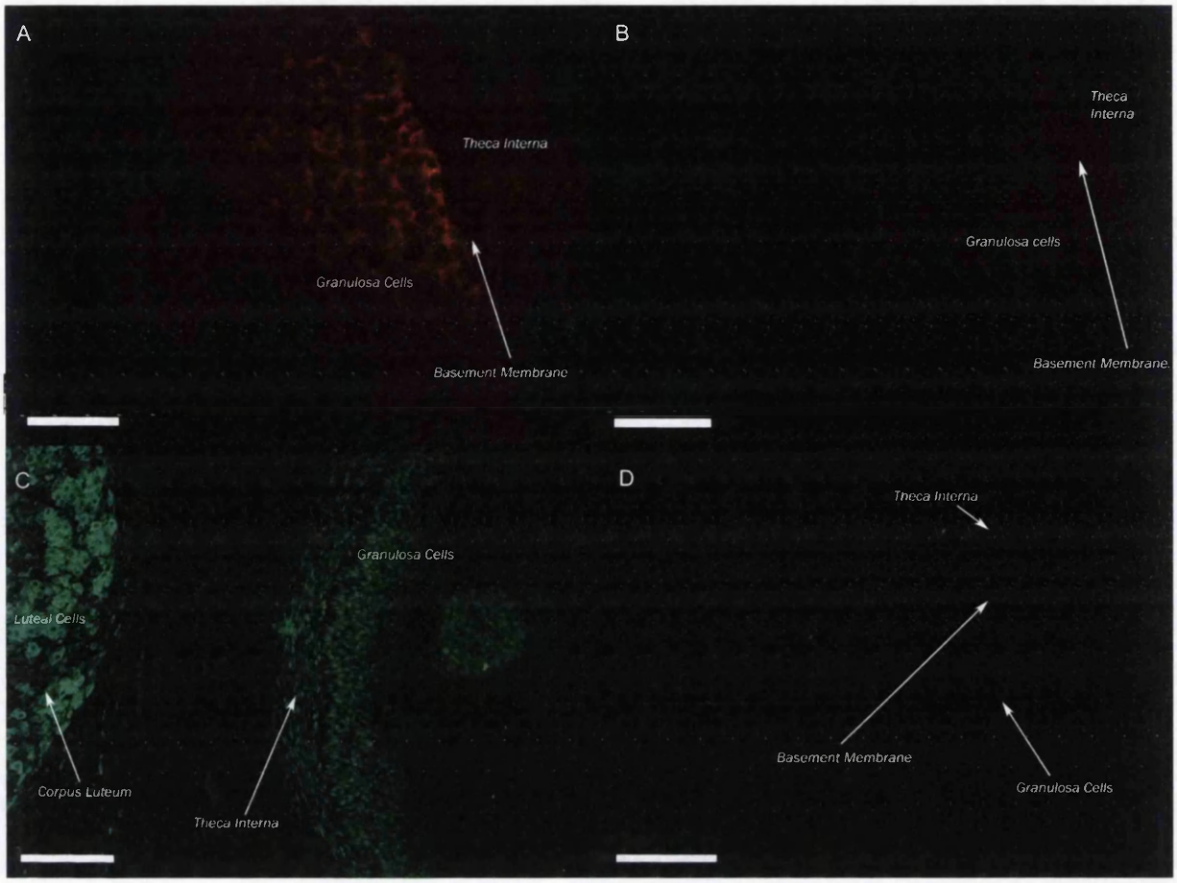


Figure 4.4 Representative images of CYP19A1 (red) and HSD3B1 (green) in ovine ovarian follicles from a control ewe aged 10 months. (**A**) CYP19A1 is localised to the granulosa cells (labelled), while no positive staining is apparent in the theca cells of the follicle. (**B**) CYP19A1 negative control. (**C**) HSD3B1 is localised to theca and granulosa cells (labelled), and to the luteal cells of the *corpus luteum*. (**D**) HSD3B1 negative control. Various follicle features are labelled. Individual scale bars are shown for each individual image. Scale bars on each image represent 100μm.

4.4 Discussion

This study is the first to have illustrated that androgenisation by TP programs antral follicle health, increasing the proportion of healthy antral follicles smaller than 1 mm in 10 month old ewes. However, the effect of a significant reduction in antral follicle atresia is lost when ewes are 22 months old. Additionally, granulosa cell aromatase steroidogenic ability is acquired at an earlier stage of follicle development, indicative of advanced follicle differentiation, as a result of prenatal androgenisation by TP when ewes are 22 months old. The implications of the results found in this study are discussed in further detail below.

4.4.1 Antral Follicle Numbers and Health

Prenatal TP treatment was associated with a reduction in the proportion of atretic antral follicles from 10 month old sheep, however, by 22 months of age this improvement in follicle health was no longer evident. However, there was a relatively large proportion (12/52) of follicles in ovaries recovered from 22 month old TP ewes where histological

atresia was not confirmed by activated CASP3 expression, in comparison to control ewes, where only 4/30 follicles classified as atretic were negative for CASP3 staining. Therefore, the subjective histological evaluation appeared to overestimate atresia, particularly within the TP-treatment group. Thus, if CASP3 expression is taken as the benchmark of atresia in follicles, then 37/77 (48%) of TP follicles should be considered healthy compared with 13/39 (33%) of control follicles. This clearly increases the proportion of healthy antral follicles in TP ovaries similar to what is seen in 10 month old ewes, although the increase was not enough to achieve statistical significance. Therefore, the loss of improved follicle health at 22 months of age in prenatally TP-treated ewes may relate to the methodology used to determine follicle health. Thus, androgenisation by TP appears to prevent apoptosis and enhances survival of early gonadotrophin-independent antral follicles, and, if this continues to later stages of development, could also explain the persistent growth of large follicles observed in ovaries from TP-treated ewes (Manikkam *et al.*, 2006; Dumesic *et al.*, 2007; Steckler *et al.*, 2007a). Prenatal androgenisation by TP improving follicle health in our study with Dorset ewes is in contrast with results obtained from the Suffolk model, where the additive effects of testosterone's oestrogenic and androgenic action increased the absolute number of healthy antral follicles (both <1 mm in diameter and those >1 mm in diameter), but not the proportion of healthy antral follicles in 10 month old sheep (Smith *et al.*, 2008). Discrepancies in the results obtained between the two studies could be as a result of the different breeds of sheep used in the study (Dorset vs Suffolk), and the fact that the Dorset breed is more susceptible to the programming actions of testosterone (Sharma *et al.*, 2002; Unsworth *et al.*, 2005; Manikkam *et al.*, 2006; Manikkam *et al.*, 2008). Additionally, in Smith's study, ovaries were collected in early-mid January, which is still in the breeding season for this breed (Robinson & Karsch, 1984) and the reproductive cycles were synchronised by prostaglandin F2α . In contrast, the ovaries from the 10 month old Dorset ewes in the current study were collected in late February (out of the breeding season) and without synchronisation of reproductive cycles.

As seen in **Chapter 3**, abnormally large antral follicles from prenatally TP-treated ovaries seem to have acquired the ability to avoid atresia causing follicles to grow for longer and attain larger maximum sizes in both breed models. However, the observation of more healthy small antral follicles, when Suffolk androgenised ewes are 10 months of age, may also be due to enhanced recruitment leading to a larger proportion of growing follicles within the follicle pool, and thus higher absolute numbers of growing antral follicles. Evidence for prenatal testosterone enhancing recruitment exists in both the Dorset (Forsdike *et al.*, 2007) and Suffolk (Steckler *et al.*, 2005; Smith *et al.*, 2008) model, and

altered recruitment is facilitated by androgenic programming (Smith *et al.*, 2008). Unfortunately, as quarter ovarian sections were used in our study using the Dorset model it was not possible to determine accurately whether there were absolutely more antral follicles within each ovary as a result of prenatal androgenisation by TP. Therefore, future studies should use the whole ovary as the starting material and use a stereological approach, as described (Smith *et al.*, 2008), to determine if increased follicle survival also results in an increase in the total number of healthy antral follicles. This would also enable determination of the effect of breed of sheep on follicle recruitment in prenatally androgenised ewes. Future studies should also determine whether it is testosterone's oestrogenic or androgenic action that results in improved antral follicle health within the Dorset breed. Therefore, prenatal androgenisation should be achieved using the non-aromatisable precursor DHT in order to delineate the oestrogenic and androgenic effects of prenatal TP on antral follicle health.

At 10 months of age androgenisation increased the proportion of healthy follicles 0.2-1 mm in diameter (gonadotrophin independent stage (Scaramuzzi *et al.*, 1993)). Thus, it appears that prenatal programming by TP improves follicle health at a specific stage of antral follicle growth, and this improved follicle health may be independent of gonadotrophin (FSH) status. Given the altered endocrine environment of increased LH (Sarma *et al.*, 2005) and increased insulin concentrations (Recabarren *et al.*, 2005) seen in prenatally androgenised ewes, this may directly or indirectly (via effects on other follicles) support (small) antral follicles in TP ewes. Insulin has been shown to increase estradiol production in cultured bovine granulosa cells from small antral (1 to 4 mm) follicles (Armstrong *et al.*, 2002), demonstrating a direct action on follicle function, while oestradiol, in turn, is a survival factor (Rosenfeld *et al.*, 2001). Additionally, insulin treatment has been shown to increase the number of follicles greater than 3 mm in diameter after ovulation in sheep (Hinch & Roelofs, 1986), and the addition of insulin also increases the number of small follicles in pigs (Matamoros *et al.*, 1991). Thus, increased concentrations of peripheral insulin could promote oestradiol production, and the anti-apoptotic actions of oestradiol may result in improved antral follicle health. More small antral follicles (0.2-1 mm) will then continue to grow and reach sizes normally indicative of the FSH dependent stage of development. The proportion of healthy antral follicles larger than 1 mm is much lower, and unchanged in androgenised ewes (our study) but this may lead to more healthy antral follicles > 1 mm if absolute numbers are increased (Smith *et al.*, 2008). Average concentrations of FSH are unchanged (Sarma *et al.*, 2005), and may

be able to support a larger number of FSH-dependent follicles, but transient FSH elevations and follicle wave growth have not been monitored in androgenised ewes.

A higher proportion of larger antral follicles (>1-2 and >2 mm in diameter) were seen as ewes grew older in both treatment groups, which mirrors that seen in women (Broekmans *et al.*, 2007; Broekmans *et al.*, 2009). Additionally, a higher proportion of larger antral follicles (>1-2 and >2 mm) were classified as healthy when ewes were 22 months of age in both treatment groups. Thus, it appears that the effects of improved follicle health by prenatal androgenisation in small antral follicles (0.2-1 mm) are lost as sheep age and controls "catch up" with their TP-treated counterparts. This could be as a result of enhanced follicle depletion present in the Suffolk TP-treated ewe (Smith *et al.*, 2008) leading to fewer antral follicles at 22 months of age, however this does not explain improved follicle health.

However, the survival or anti-apoptotic mechanisms programmed by the effects of prenatal TP, which improve follicle health in smaller than 1 mm and in large antral follicles, are unknown. Therefore, expression of pro- and anti-apoptotic factors may be altered in smaller sized FSH-independent follicles. Both CASP3 and BAX play roles in small antral follicle atresia (Mani *et al.*, 2010), thus future studies into the investigation of follicle atresia should use semi-quantitative immunohistochemistry to determine if the protein levels of either factor are altered by prenatal androgenisation.

4.4.2 Antral Follicle Steroidogenesis Capacity

HSD3B1 was present in thecal cells of antral follicles, from 0.24 mm in diameter as has been previously determined (Logan *et al.*, 2002). Androgenisation by TP did not increase the proportion of antral follicles immunoreactive for HSD3B1 in thecal cells. By 22 months of age, the proportion of follicles with HSD3B1 present in the theca cells in controls and TP-treated ewes was reduced compared to ewes aged 10 months. We are unaware of any previous studies that have investigated the proportion of antral follicles immunoreactive for HSD3B1 in theca cells in aging sheep. Thus, from our results, it would appear that in sheep, as the ovary ages, the proportion of antral follicles synthesising progesterone in the theca decreases.

Granulosa cells from small antral follicles were immunoreactive for both HSD3B1 and CYP19A1 (Conley *et al.*, 1995; Logan *et al.*, 2002) and no thecal cells showed immunoreactivity for CYP19A1 as expected (Logan *et al.*, 2002). Expression of HSD3B1

in granulosa cells is a follicle differentiation marker, as its expression was only determined in selected dominant follicles (Bao & Gaverick, 1998; Fortune et al., 2001). As more early antral follicles are healthy, and large antral follicles show much enhanced progesterone synthesis, we asked whether the HSD3B1 enzyme was already expressed in granulosa cells from smaller antral follicles in TP-treated ovaries which would demonstrate premature follicular differentiation. Androgenisation by TP was associated with small antral follicles less than 1 mm in diameter showing immunoreactivity for HSD3B1 in granulosa cells from 10 month old ewes. However, due to the small number of follicles analysed, no statistical difference was achieved between the two treatment groups in the proportions of early antral follicles immunoreactive for HSD3B1 in granulosa cells. Thus, prenatal androgenisation by TP did not lead to premature differentiation using HSD3B1 protein localisation as the differentiation marker.

Prenatal TP treatment was associated with a higher proportion of small antral follicles (<1 mm in diameter) immunoreactive for CYP19A1 in granulosa cells in the older age group of sheep. In normal sheep, the aromatase protein is not observed in granulosa cells of any follicles ≤3 mm in diameter (Logan *et al.*, 2002), but is present in follicles between 3.5 and 5 mm in diameter (Huet *et al.*, 1997), and as a result can be used as a differentiation marker for terminal follicle development. This suggests that early antral follicles from 0.2 mm in diameter from prenatally TP-treated ewes are already further differentiated than their control counterparts of a similar size. Thus, it appears that prenatal androgenisation by TP results in premature follicle differentiation during the early stages of antral follicle development, specifically at the small antral follicle stage, and continues through to the large antral stage. Previous evidence that prenatal androgenisation by TP enhances follicle differentiation at 10 months of age exists within the Suffolk breed, where follicles exhibited antrum formation at smaller sizes (Smith *et al.*, 2008).

Oestradiol production by granulosa cells, both *in vivo* (McNatty *et al.*, 1985; Campbell *et al.*, 1998) and *in vitro* (Campbell *et al.*, 1996) is stimulated by FSH, whereas withdrawal of FSH support *in vivo*, in the absence of LH, leads to an acute decrease in oestradiol secretion and atresia of cohort and dominant follicles (Campbell *et al.*, 1999). It is well established that it is mainly the action of FSH that induces the expression of CYP19A1 in granulosa cells (Gore-Langton & Dorrington, 1981; Richards, 1994) and, in regard to follicle development, this induction usually occurs in the FSH-dependent stage (Campbell *et al.*, 2003; Evans, 2003a). However, there is no evidence to suggest that it is the action of FSH that increases the survival of early antral follicles, as follicles of this size are

considered FSH-responsive for proliferation of cells, but not FSH-dependent for enhanced growth and differentiation (Scaramuzzi *et al.*, 1993) Only granulosa cells within large antral follicles show CYP19A1 protein localisation (Logan *et al.*, 2002), when follicles are FSH dependent. It appears that androgenisation induces aromatase expression within early antral follicles considered independent of FSH and, thus, some other factors, such as IGF-1 (Spicer *et al.*, 1993; Costrici *et al.*, 1994; Richards, 1994) and insulin (Christman *et al.*, 1991; Armstrong *et al.*, 2002) could induce granulosa cell CYP19A1 expression.

Early antral follicles are able to synthesise oestradiol given that the required steroidogenic enzymes are expressed in theca cells of preantral follicles (Conley *et al.*, 1995; Logan *et al.*, 2002) and, thus, the precursor required for oestradiol synthesis is available. While increased systemic oestradiol concentrations in prenatally androgenised ewes (by TP) seen in **Chapter 3** are most likely due to increased DF and SF capacity for steroidogenesis, premature differentiation of granulosa cells of antral follicles less then 1 mm in diameter may also add to the ovarian secretion of oestradiol.

It is difficult to speculate about how advanced follicle development arises in prenatally androgenised ewes. Previous studies of the effects of continual exposure to elevated concentrations of gonadotrophins on antral follicle development in Booroola Merino sheep determined that follicles synthesise steroids and mature at smaller diameters compared to those exposed to normal levels of FSH and LH (McNatty & Henderson, 1987). However, in the androgenised Suffolk ewe, FSH concentrations remain static and low (Sarma *et al.*, 2005) indicating that small antral follicles may have an inherent high FSH-responsiveness or an FSH-independent mechanism for growth and differentiation (induction of oestradiol production). Such a FSH-independent mechanism for growth and differentiation of small antral follicles may involve other growth factors such as insulin or IGF-1 being present in higher concentrations within the ovary in TP-treated ewes. It is also possible that the advanced follicle differentiation is a result of elevated peripheral concentrations of LH observed in prenatally TP-treated ewes (Robinson *et al.*, 1999; Sarma *et al.*, 2005). In order to investigate antral follicle differentiation and their ability to respond to FSH, insulin, IGF-1 and LH in TP-treated animals, future studies should use immunohistochemistry to investigate both the presence and amount of FSHR, IGF-1 and insulin receptor, and LHR present in granulosa cells of early antral follicles.

In conclusion, the results from this study demonstrate that androgenisation by TP programs antral follicle health and follicle development such that survival of early antral follicles is

enhanced, and an important aspect of follicle differentiation, specifically aromatase acquisition in granulosa cells, is achieved at smaller sizes. It remains to be seen whether this is related to advanced FSH- or indeed LH-dependence in small antral follicles, if it is the androgenic or oestrogenic action of testosterone that results in premature follicle differentiation, or whether it is the action of elevated systemic LH that drives this dysfunction of antral follicle development.

CHAPTER 5. EFFECT OF PRENATAL TESTOSTERONE TREATMENT ON GRANULOSA CELL GENE EXPRESSION IN DOMINANT AND SUBORDINATE FOLLICLES OF ADULT EWES

5.1 INTRODUCTION

As demonstrated in **Chapter 3**, prenatal androgenisation by testosterone propionate (TP) treatment in sheep during a specific critical period in gestation results in follicular abnormalities within the largest follicles designated the dominant (DF) and subordinate follicle (SF) based on intrafollicular oestradiol. Specifically, DF and SF from TP-treated ovaries show enhanced growth and increased oestradiol and progesterone synthesis. The mechanism by which this increase in growth and steroidogenesis arises is unknown. An increase in the number of granulosa cells, as a result of reduced apoptosis and/or increased proliferation, could explain increased growth. Such abnormal proliferation and reduced apoptosis within persistent follicles leading to polycystic ovaries has been shown in rats (Salvetti *et al.*, 2009). Additionally, an increase in follicular fluid oestradiol and progesterone in DF and SF implies that these follicles are highly differentiated, as both characteristics sequentially (first enhanced oestradiol, then enhanced progesterone synthesis, (Sunderland *et al.*, 1994; Evans *et al.*, 2000)) are markers of preovulatory follicle differentiation (Webb *et al.*, 2003). Thus, changes in the expression profiles of follicle growth, survival and differentiation genes would help explain the mechanisms by which prenatal TP androgenisation programs adult antral follicle function.

In order to characterise further the follicular and cellular abnormalities underlying excessive and prolonged large antral follicle growth and enhanced steroidogenesis in TP-treated ewes, granulosa cell mRNA expression for genes known to regulate gonadotrophin responsiveness, steroidogenesis, cell proliferation, cell survival or apoptosis in growing antral follicles before and after dominant follicle selection (Bao *et al.*, 1997; Mihm & Austin, 2002; Fortune *et al.*, 2004; Webb & Campbell, 2007) were studied. The genes investigated included those conveying gonadotrophin responsiveness, luteinising hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR exon 4/5 and FSHR exon 9/10), and the steroidogenic enzymes aromatase (CYP19A1) and 3-beta hydroxysteroid dehydrogenase (HSD3B1). We expected that, as a result of prenatal programming increasing steroidogenesis, granulosa cell CYP19A1 and HSD3B1 mRNA expression would be upregulated by prenatal androgen excess. Additional genes studied included those encoding the FSH dependent somatic cell differentiation markers inhibin alpha (INHA), inhibin beta subunit A (INHBA) and follistatin (FST), the cell proliferation marker Cyclin D2 (CCND2), the cell differentiation marker, macrophage migration inhibitory factor (MIF) and the cell differentiation marker betaglycan (BGCAN). The final

group of genes investigated encompassed those encoding proteins with either pro-apoptotic or anti-apoptotic function. The pro-apoptotic markers included caspase-3 (CASP3) and BCL2 associated X protein (BAX), while myeloid cell leukemia sequence 1 (MCL-1) is anti-apoptotic (Johnson & Bridgham, 2002). As a result of the stimulation of growth in large antral follicles by prenatal androgenisation (**Chapter 3**), we would expect an upregulation of the expression of anti-apoptotic genes (*MCL-1*) and a downregulation of the expression of pro-apoptotic genes (*CASP3* and *BAX*) within granulosa cells of DF and SF. To the best of our knowledge, no group has studied the granulosa cell mRNA expression profiles within DF and SF recovered from prenatally TP-treated ewes. This investigation will, therefore, facilitate the molecular characterisation of granulosa cells within DF and SF recovered from TP-treated ewes. A brief introduction to each gene together with a description of its function is given below.

Gonadotropin responsiveness (LHR, FSHR exon 4/5 and FSHR exon 9/10)

The gonadotrophins, FSH and LH, are the primary endocrine hormones that regulate follicle growth and development, particularly during the terminal stages of follicular growth (Phillips *et al.*, 1994; Campbell *et al.*, 1999). The receptors for the gonadotrophins, luteinising hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR), are required for gonadotrophin action (Simoni *et al.*, 1997; Dufau, 1998). Follicle stimulating hormone receptor and LHR are expressed in granulosa cells of large, highly differentiated antral follicles (Hillier, 2001). Changes in the pattern of expression of mRNA for gonadotrophin receptors and steroidogenic enzymes within follicular cells appear to be closely linked to changes in peripheral concentrations of gonadotrophins and steroids (Webb *et al.*, 2003). Any changes in gonadotrophin receptor transcript levels are likely to alter gonadotrophin responsiveness (Mihm *et al.*, 2006). Both LHR and FSHR genes encode G-coupled protein receptors (Simoni *et al.*, 1997; Dufau, 1998; Hillier, 2001). The post-receptor signalling systems that relay gonadotrophin action into the cell nucleus rest mainly on adenylyl cyclase, cAMP production and activation of protein kinase A (PKA) (Richards, 1994; Richards *et al.*, 1998). Stimulation by FSH via FSHR and LH via LHR increases intracellular cAMP formation and activation of genes required for proliferation and differentiation (Hillier, 2001). The effect of prenatal programming by excess androgens on the ability of granulosa cells from DF and SF to respond to the gonadotrophins is unknown. The ability of granulosa cells to respond to gonadotrophins is likely to be abnormal given the increased LH pulsatility within androgenised ewes (by TP) (Sarma *et al.*, 2005; Veiga-Lopez *et al.*, 2009). Growing DF show an upregulation of *LHR* and downregulation of *FSHR* mRNA in granulosa cells (Mihm *et al.*, 2006), and

preovulatory follicles in the follicular phase (which may be similar to the large persistent follicles seen in prenatally TP-treated ewes), show increased LH- but decreased FSH-receptor binding (Ireland & Roche, 1983b). Thus, from these previous results, it is speculated that prenatal androgenisation would up regulate granulosa cell *LHR* expression, but down regulate *FSHR* expression within large antral follicles. This study investigated the granulosa cell expression levels of two FSHR amplicons. The *FSHR* exon 4/5 primer combination detects mRNA encoding the extracellular ligand binding portion of the receptor, which includes alternate transcripts not encoding the functional FSH receptor (Simoni *et al.*, 1997). In comparison, the amplicon using the *FSHR* exon 9/10 primer combination encodes the intracellular domain of FSHR (Simoni *et al.*, 1997). Although exon 10 is fundamental for signal transduction, it is not necessary for ligand binding (Gromoll & Simoni, 2005). Both transcripts were investigated to determine the expression profiles of both the ligand binding and signal transduction domains of the FSHR.

Steroidogenesis (CYP19A1 and HSD3B1)

The steroidogenic enzymes aromatase (CYP19A1) and 3 beta-hydroxysteroid dehydrogenase (HSD3B1) are responsible for oestradiol and progesterone production, respectively (Conley *et al.*, 1995; Gruber *et al.*, 2002); Amsterdam *et al.*, 2003). Follicular steroidogenesis requires both theca and granulosa cells for the production of steroids, working together in cooperation (Drummond, 2006). Progesterone, androgens and oestrogen are synthesised by the ovary in a sequential manner, with each serving as substrate for the subsequent steroid in the pathway. Such pathways have not been studied in antral follicles from androgenised ewes. In the sheep, *CYP19A1* is present solely in granulosa cells from follicles greater than 3.5 mm in diameter (Huet *et al.*, 1997; Logan *et al.*, 2002), while *HSD3B1* is localised to both granulosa and theca cells in similarly sized follicles (Conley *et al.*, 1995; Logan *et al.*, 2002). Granulosa cell *CYP191A* and *HSD3B1* expression in ovine DF is high compared to developing follicles that are smaller (3 to 5 mm) in diameter (Duggavathi *et al.*, 2006). Increased expression of both of these enzymes within granulosa cells of DF and SF could explain the higher intrafollicular fluid oestradiol and progesterone concentrations observed in TP-treated ewes (**Chapter 3)** due to increased steroid synthesizing capacity.

FSH-dependent cell differentiation (INHA, INHBA and FST)

Abnormal differentiation of DF and SF by prenatal androgenisation through TP was also investigated by determining granulosa cell expression of inhibin alpha *(INHA)* subunit, inhibin beta subunit (*INHBA*) and follistatin (*FST*). The *INHA* gene encodes the α-subunit

necessary to form the peptide inhibin whose role, in synergy with oestradiol, is to inhibit tonic FSH secretion from the pituitary by inhibiting the stimulatory actions of activins (Mann *et al.*, 1990; Baird *et al.*, 1991). As detailed earlier, circulating concentrations of inhibin A are highest during the late follicular phase (Campbell *et al.*, 1990; Findlay *et al.*, 2000), as a result of production from large preovulatory oestrogenic follicles. *INHBA* encodes the βA-subunit required to form either inhibin A (α-βA subunits) or two of the three activin peptides which are generated following dimerisation of β subunits, activin A (βA-βA) and activin AB (βA-βB) (Webb *et al.*, 1994; Knight & Glister, 2001). Granulosa cell *INHBA* expression increases as follicle size increases, the DF producing ever increasing amounts throughout its growth until ovulation or regression (Roberts *et al.*, 1993). FST binds to and bio-neutralises the actions of activin (Ying, 1988) which in an indirect way promotes luteinisation as activin inhibits luteinisation (reviewed, Knight & Glister, 2006). *FST* is present in granulosa cells of antral follicles and as the follicle matures expression increases and follicular fluid concentrations also increase, while during atresia granulosa cell *FST* expression is reduced (Roberts *et al.*, 1993; Lin *et al.*, 2003; Glister *et al.*, 2006). Any changes in the expression levels of these genes will also affect FSH secretion and may affect FSH-dependent differentiation of antral follicles within prenatally androgenised ewes.

Cell proliferation and differentiation (CCND2 and MIF)

The protein encoded by Cyclin D2 (*CCND2*) gene belongs to the highly conserved Cyclin family, whose members are characterised by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of Cyclin dependent kinases (CDK's), CCND2 forming a complex with, and functioning as, a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition (Sicinski *et al.*, 1996) and, thus, promotes cell proliferation. Knockout *CCND2* studies in mice suggest essential roles of this gene in ovarian granulosa and germ cell proliferation (Sicinski *et al.*, 1996). Both FSH and oestradiol have been shown to induce *CCND2* expression in granulosa cells in mice (Robker & Richards, 1998). CCND2 has been shown to be an indirect marker of follicle survival, as in the bovine, *CCND2* expression within granulosa cells is upregulated within DF when compared to SF (Mihm *et al.*, 2008). In addition, CCND2 is downregulated when follicles luteinise in response to the LH surge, therefore, when cell proliferation ceases during final differentiation before ovulation (Sicinski *et al.*, 1996; Robker & Richards, 1998; Hernandez-Gonzalez *et al.*, 2006). Thus, reductions in *CCND2* granulosa cell expression may mark follicle differentiation within the ruminant follicle, demonstrating a shift from cell proliferation to differentiation.

Macrophage Migration Inhibitory Factor (MIF) was discovered as an activated T-lymphocyte-derived protein that inhibits the random migration of macrophages *in vitro* but is also secreted by macrophages in response to cytokine stimulation (Nishihira, 1998; Wada *et al.*, 1999). MIF exerts a variety of biological functions and is expressed in cells other than those belonging to the immune system (Wada *et al.*, 1999). Various possible roles of MIF in different aspects of reproduction, such as ovulation, blastocyst implantation, and embryogenesis have been identified (Nishihira, 1998). MIF is expressed in the bovine CL (Bove *et al.*, 2000), providing evidence for a role for MIF in the processes of CL formation, and, thus, may be a possible marker of luteinisation (differentiation). In the ovine, MIF is localised to large luteal cells in CL and granulosa cells of primary, secondary and tertiary follicles (Rath-Coursey *et al.*, 1999). Similar to CCND2, in the bovine, *MIF* is a follicle dominance marker gene, with mRNA levels elevated in the DF compared to the SF (Mihm *et al.*, 2008). Any changes in the expression profile of *CCND2* and *MIF* will provide an insight into whether prenatal androgenisation by TP alters granulosa cell proliferation and differentiation in large antral follicles from adult ewes.

Differentiation and apoptosis – Betaglycan (BGCAN) (TGFbeta III receptor)

Transforming growth factor (TGF)-beta is a multifunctional cytokine that modulates several tissue development and repair processes, including cell differentiation, cell cycle progression, cellular migration, adhesion, and extracellular matrix production (Massague, 1990; Moulton, 1994; Clark & Coker, 1998). Previous studies in sheep have shown that ovarian cells express both TGFß mRNA and proteins involved in numerous ovarian functions (Findlay *et al.*, 2001; Knight & Glister, 2003). Similar to other growth factors, the signals of TGFß are mediated by the interaction with various cell surface receptors (Lin & Moustakas, 1994; Chang *et al.*, 2002). One such receptor is the TGFbeta III receptor, also known as betaglycan (BGCAN). In addition to serving as a co-recptor for TGFβ, betaglycan also increases the affinity of inhibins for the activin type IIA receptor, thereby blocking activin binding and signalling through this receptor (Lewis *et al.*, 2000), possibly playing an important role in regulating follicular growth (Forde *et al.*, 2008). *BGCAN* expression is induced by FSH in cultured human granulosa cells (Liu *et al.*, 2003), and may be an indirect differentiation marker as *BGCAN* mRNA expression is down regulated in the DF compared to the SF (Evans *et al.*, 2004; Forde *et al.*, 2008). Any changes in the expression pattern of *BGCAN* will provide an insight into the differentiation status of granulosa cells of DF and SF of prenatally TP- ewes. This will also allow the estimation of

the relative roles of activin A and inhibin A within such follicles, as any BGCAN upregulation may increase inhibin's ability to antagonize activin actions.

Cell survival/apoptosis (MCL-1, CASP3 and BAX)

Increased expression of anti-apoptotic genes together with decreased expression of pro-apoptotic genes, thus reducing granulosa cell death, would result in follicles failing to regress and continuing to grow achieving larger follicle sizes. Such enhanced growth of follicles from prenatally androgenised ewes was seen in **Chapter 3**. As stated previously granulosa cell death during follicular atresia results from apoptosis (Tilly *et al.*, 1991; Hsueh *et al.*, 1994a; Rolaki *et al.*, 2005). Apoptosis is the most characterised event in atretic follicles and is a highly regulated, physiological process (Jolly *et al.*, 1994; Jolly *et al.*, 1997a, b). Apoptosis occurs at all stages of follicle development and, within the follicle, the first sign of atresia is apoptotic granulosa cells (Kugu *et al.*, 1998; Irving-Rodgers *et al.*, 2001). Many prosurvival and proapoptotic proteins are involved in apoptosis (Johnson & Bridgham, 2002; Rolaki *et al.*, 2005). The BCL-2 gene family comprises a large family of proteins, whose individual members can act in either an anti-apoptotic or pro-apoptotic manner when differentially expressed (Hsu & Hsueh, 2000). One such member is myeliod cell leukaemia factor 1 (MCL-1) which is an anti-apoptotic protein (Sato *et al.*, 1994; Krajewski *et al.*, 1995; Hsu & Hsueh, 2000). Using the yeast 2 hybrid system MCL-1 was identified as the main ovarian antiapoptotic BCL-2 protein (Hsu & Hsueh, 2000). MCL-1 is expressed in the developing follicle throughout gestation, particularly in oocytes, granulosa cells and in the adult ewe in granulosa lutein cells and is considered to have an important follicular developmental role (Krajewski *et al.*, 1995; Hartley *et al.*, 2002). Within bovine dominant follicles *MCL-1* granulosa cell expression is upregulated (Evans *et al.*, 2004) and, thus MCL-1 also acts as a DF survival marker gene. Its antagonist, BAX, is also expressed in granulosa cells during follicular development (Choi *et al.*, 2004). Immunohistochemical localization of BAX in the human ovary reveals abundant expression in granulosa cells of early atretic follicles, whereas BAX protein is extremely low or non-detectable in healthy or grossly-atretic follicles (Kugu *et al.*, 1998). The major downstream effector of apoptosis, for both the intrinsic and extrinsic apoptotic pathways is caspase-3 (CASP3) (Matikainen *et al.*, 2001; Johnson & Bridgham, 2002), which activates endogenous nucleases responsible for DNA fragmentation (Thornberry & Lazebnik, 1998). Studies using *CASP3* gene knockout mice determined that CASP3 is functionally required for granulosa cell apoptosis during follicular atresia (Matikainen *et al.*, 2001). Granulosa cells from healthy follicles possess almost exclusively the inactive (unprocessed) form of CASP3, whereas granulosa cells from atretic follicles demonstrate

increased concentrations of activated CASP3 (reviewed, Johnson & Bridgham, 2002; Feranil *et al.*, 2005) and increased expression of the CASP3 gene within human granulosa cells (Izawa *et al.*, 1998) and ovine luteal cells (Rueda *et al.*, 1999). In PCOS patients granulosa cell apoptosis is reduced through lower ovarian CASP3 mRNA and activated protein expression (Das *et al.*, 2008). Thus, any changes in expression of these three anti- and pro-apoptotic genes (*MCL-1*, *BAX* and *CASP3*) will provide an insight into the effect of prenatal testosterone on the programming of apoptosis in granulosa cells of DF and SF from adult ewes.

In summary, increased growth, enhanced differentiation and steroidogenesis within the largest antral follicles may be due to differences in the expression of genes involved in gonadotrophin responsiveness, steroidogenesis, cell differentiation, cell survival factors and apoptosis. The aim of this study was to determine the effect of prenatal androgenisation by excess TP on the mRNA expression levels of a selected number of genes mentioned above within granulosa cells of DF and SF.

5.2 Materials and Methods

5.2.1 Animals and Prenatal Treatment

Ewes were androgenised using an established model (Robinson *et al.*, 1999; Steckler *et al.*, 2005) and euthanased at 10- (Year Group 3, Control = 7, TP = 10, Year 4, Control = 7, TP = 6 and Year Group 5, Control = 14, TP = 15, DHT = 8) and 12-months of age (Year Group 2, Control = 7, TP = 6 ewes) for ovary collection.

5.2.2 Isolation of Granulosa Cells

Ovaries from control and TP-treated ewes were harvested at 10 and 12-months of age and the largest antral follicles over 3.5 mm (controls) or from 5 mm (TP androgenised ewes) were identified from surface measurements using callipers, excised and the follicular fluid collected as described in section 2.4. Granulosa cells from individual follicles were collected, washed and stored as described in section 2.4 prior to RNA extraction.

5.2.3 Molecular Biology

RNA was extracted from granulosa cells recovered from DF and SF using the TRIzol reagent and DNase I treated. Following DNase I treatment, RNA quantity and quality was determined by spectrophotometry. SuperScript III First-Strand Synthesis System (Invitrogen Ltd, Paisley, UK) was used to synthesise cDNA from 500 ng of total RNA isolated from granulosa cells of individual follicles. Following reverse transcription, cDNA quality and quantity was also determined by spectrophotometry. The semi-quantitative

real-time PCR (qPCR) method was used to measure gene expression within the granulosa cells and validation studies were conducted to ensure that the amplification efficiencies of the genes of interest were similar to the references gene. Precise details of all these methods are given in section 2.7. The qPCR reactions were performed in duplicate, and a reagent blank was included within each plate to detect contamination by genomic DNA. The sequences of the forward and reverse primer for each gene of interest used in the study are shown Table 4.1. We quantified *LHR*, *FSHR* exon 4/5, *FSHR* exon 9/10, *CYP19A1*, *HSD3B1*, *INHA*, *INHBA*, *FST*, *CCND2*, *MCL-1*, *BGCAN*, *MIF*, *CASP3* and *BAX* mRNA expression using the comparative C_T (cycle threshold) method (Schmittgen & Livak, 2008) and we calculated gene expression relative to the reference gene (*ACTB*). The expression profiles of the genes of interest used in the study were (in some cases) investigated only in certain years, depending on the availability of cDNA (year group 4). Table 5.2 lists the year groups that were studied for each gene of interest.

5.2.4 Statistical analyses

For individual follicle gene expression profiles within granulosa cells of DF and SF, a GLM with year group (2-5), prenatal treatment (Control and TP) and follicle classification (DF and SF) as the fixed factors was used to determine any influences on follicle mRNA expression. In addition, any logical interactions between factors were included in the model. If there were more than 2 levels to a factor, a Tukey's posthoc test was used to determine significant differences between levels within that factor. Because the factor 'animal' is known to contribute to the variation in results, but could not be included in the GLM analyses, a generalised least square (GLS) model was also applied to the data with the same fixed factors as for the GLM analyses, but with 'animal' included as a random factor to determine whether this altered the outcome of GLM analyses. The 'animal' effect was significant, but overall did not alter the results from the GLM analysis. However, the GLS analysis did not consider interactions and only had limited post hoc analyses. Because of this and the agreement between GLM and GLS analyses, the results from the GLM analyses with interactions and posthoc tests are presented. In addition to the GLM results, GLS analysis showed that follicle classification tended to influence levels of an interesting subgroup of FSH-dependent transcripts, and these results will also be presented.

Gene	Accession No.	Primer	Sequences 5' to 3'	Amplicon (bp)	Tm
Housekeeping gene					
ACTB	U39357	F	GCCCTGAGGCTCTCTTCCA	73	79°C
		R	GGAATTGAAGCTAGTTTCGTGAAT		
Steroidogenesis					
CYP19A1	AJ012153	F	GGAGAGGAAACCCTTATTATTAGCAAA	96	75°C
		R	CAACCCAAGTTTACTGCCAAATC		
HSD3B1	X17614	F	CCCATTCCTTTCTGCCTACATG	78	78°C
		R	TCTTGAGAACTTGCAGTGATTGG		
Gonadotrophin Responsiveness					
FSHR Exon 4/5	L07302	F	AGGATTCGGAGACCTGGAGAA	94	77°C
		R	ATGTAGTTTGGGCAGGTTGGA		
FSHR Exon 9/10	L07302	F	TGGTCCTGTTCTACCCCATCA	110	80°C
		R	GAAGAAATCCCTGCGGAAGTT		
LHCGR	L36329	F	TTGAGCCTGGAGCATTTACAAA	109	76°C
		R	TCGTAACATCTGGAAGCTTTCG		
Cell Differentiation					
INHA	L28815	F	GCCCGAGGACCAAGATGTC	189	87°C
		R	TCCTGTCTGTCCAGTCCTGTGT		
INHBA	NM_001009458	F	GATCATCACGTTCGCGGAAT	72	80°C
		R	TCACTGCCTTCTTGGGAAATCT		
FST	M63123	F	CAGTTCATGGAGGACCGCA	122	85°C
		R	CGGCCAGTGCTGCAGC		
Proliferation/Differentiation					
CCND2	NM_001076372	F	CGCTGACCGCTGAGAAGTTAT	101	82°C
		R	GTTCCATTTCAACTTGCCCAG		
MCL-1	NM_001099206	F	GATAGTCAAACAAAGAGGCTGGGA	51	74°C
		R	TCTACACGGAAGAACTCCACAAAC		
Apoptosis					
BGCAN	XM_001253071	F	GAAAGGAATATGGAGCGGTCAC	108	76°C
		R	TCCCTATGTTGCACGTGGG		
MIF	XM_001033608	F	GCATTAGCCCGGACAGGAT	87	83°C
		R	GGCCCTCAGGCGAAGGT		
CASP3	NM_001077840	F	AGCCATGGTGAAGAAGGAATC	87	75°C
		R	CTGCAATAGTCCCCTCTGAAG		
BAX	NM_173874	F	TGAAGCGCATTGGAGATGAA	182	84°C
		R	CTTGAGCACCAGTTTGCTGG		

Table 5.1 Primer sequences for semiquantitative analysis of mRNA levels using real-time PCR. Included is the accession number and amplicon size for each gene. Primers were designed to amplify across at least one intron boundary to ensure against genomic contamination. All primers were used at a concentration of 1000nM. Tm = predicted melting temperature of the amplicon. *ACTB* – Beta Actin; *AROM* – Aromatase; *3B-HSD* – 3-beta hydroxysteroid dehydrogenase; *FSHR* – Follicle Stimulating Hormone Receptor; *LHR* – Luteinizing Hormone Receptor, *INHA* – Inhibin alpha; *INHBA* – Inhibin-beta subunit A. F – forward, R – reverse.

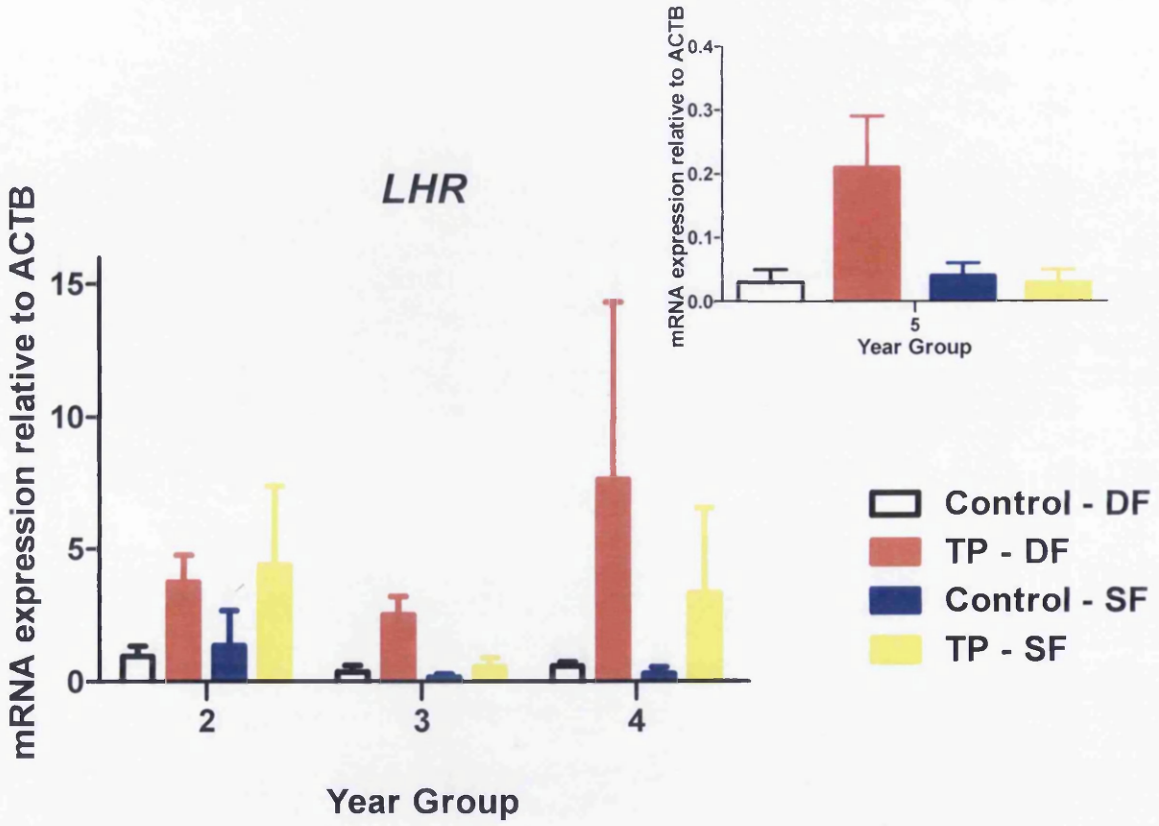
Gene of Interest	Year Groups Studied
LHR	2, 3, 4 and 5
FSHR exon 4/5	2, 3, 4 and 5
FSHR exon 9/10	2, 3 and 5
CYP19A1	2, 3, 4 and 5
HSD3B1	2, 3, 4 and 5
INHA	2 and 3
INHBA	2 and 3
FST	2, 3 and 4
BGCAN	2 and 3
CCND2	2, 3 and 5
MIF	2, 3, 4 and 5
MCL-1	2 and 3
BAX	2 and 3
CASP3	2, 3 and 5

Table 5.2 The year groups investigated for each gene of interest used within the study into the effects of prenatal androgenisation by testosterone propionate on granulosa cell mRNA gene expression from dominant and subordinate follicles.

5.3 RESULTS

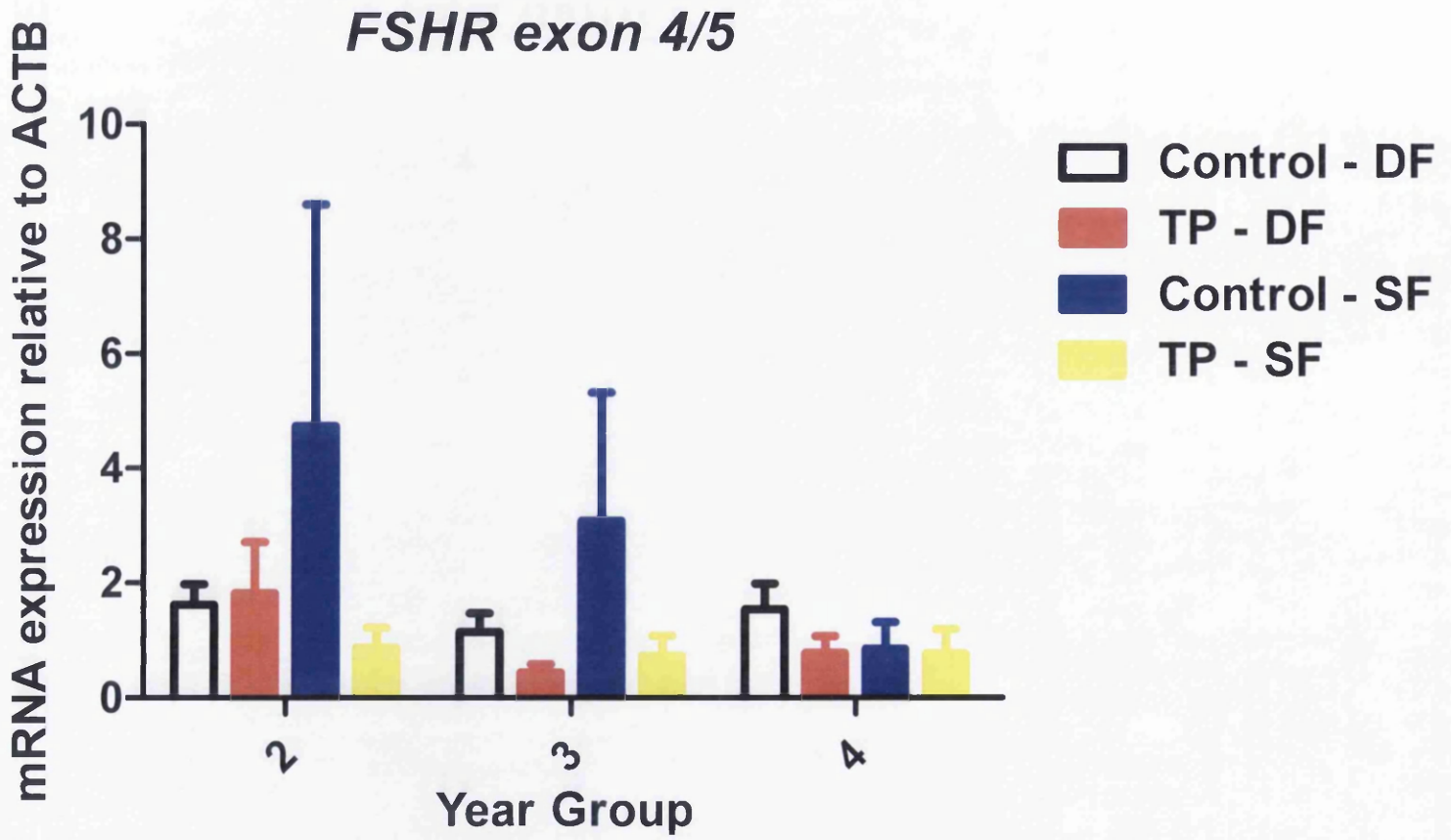
Gonadotrophin responsiveness - Expression of mRNA encoding *LHR*, *FSHR* exon 4/5 and *FSHR* exon 9/10 in granulosa cells of DF and SF.

Quantification of mRNA species in granulosa cells from DF and SF showed different mRNA levels for the gonadotropin receptor genes *LHR* and *FSHR* (using both primer combinations) between TP-treated and control ewes (Figures 5.1, 5.2 and 5.3, respectively). In granulosa cells of DF and SF, *LHR* was consistently expressed at a higher level in TP-treated ewes than in controls (Figure 5.1). *LHR* mRNA levels were also increased in granulosa cells from follicles classified as dominant compared to those classified as subordinate (Figure 5.6). Differences in the expression of *LHR* mRNA between years was apparent, with expression in year group 5 significantly different to all other year groups. While there was only a tendency for *FSHR* exon 4/5 mRNA expression to be lower in follicular granulosa cells of TP-treated ewes using the primer combination which encodes the extracellular ligand binding portions of the receptor (Figure 5.2), prenatal androgenisation by TP clearly reduced the expression of *FSHR* exon 9/10 mRNA expression in granulosa cells of DF and SF from each ewe (Figure 5.3). *FSHR* mRNA levels were similar in granulosa cells from follicles classified as dominant compared to those classified as subordinate using both primer combinations (Figure 5.6). Equally, there was no difference between each year group in *FSHR* mRNA levels using both primer combinations. Analysis tables for *LHR*, *FSHR* exon 4/5 and *FSHR* exon 9/10 mRNA granulosa cell expression within DF and SF, which list the P values determined for each factor included within the statistical model, are shown in Figures 5.1, 5.2 and 5.3 respectively.



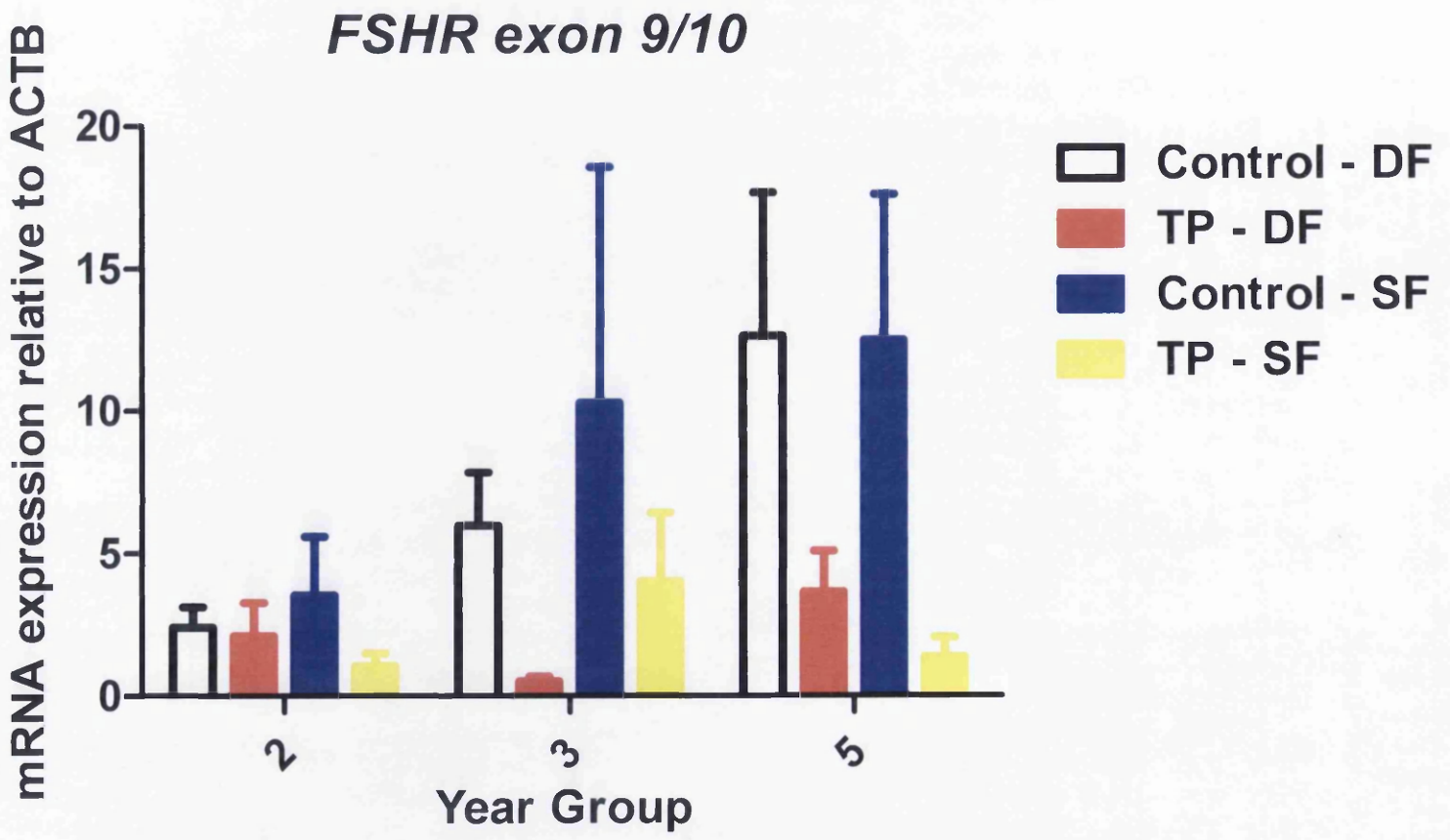
Factor	GLM P value
Year	0.000
Prenatal Treatment	0.001
Follicle Classification	0.002
Prenatal Treatment*Follicle Classification	0.354

Figure 5.1 Mean *LHR* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3, 4 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 7; TP = 6 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 4; TP = 4 follicles). *ACTB* = β-Actin. Note that the scale bar for year 5 is different from years 2, 3 and 4.



Factor	GLM P value (GLS)
Year	0.306
Prenatal Treatment	0.068
Follicle Classification	0.156 (0.090)
Prenatal Treatment*Follicle Classification	0.810

Figure 5.2 Mean *FSHR* exon 4/5 mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3 and 4. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9 and Group 4 – C = 7; TP = 5 – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5 and Group 4 – C = 6; TP = 4). *ACTB* = β-Actin.



Factor	GLM P value
Year	0.121
Prenatal Treatment	0.001
Follicle Classification	0.735
Prenatal Treatment*Follicle Classification	0.889

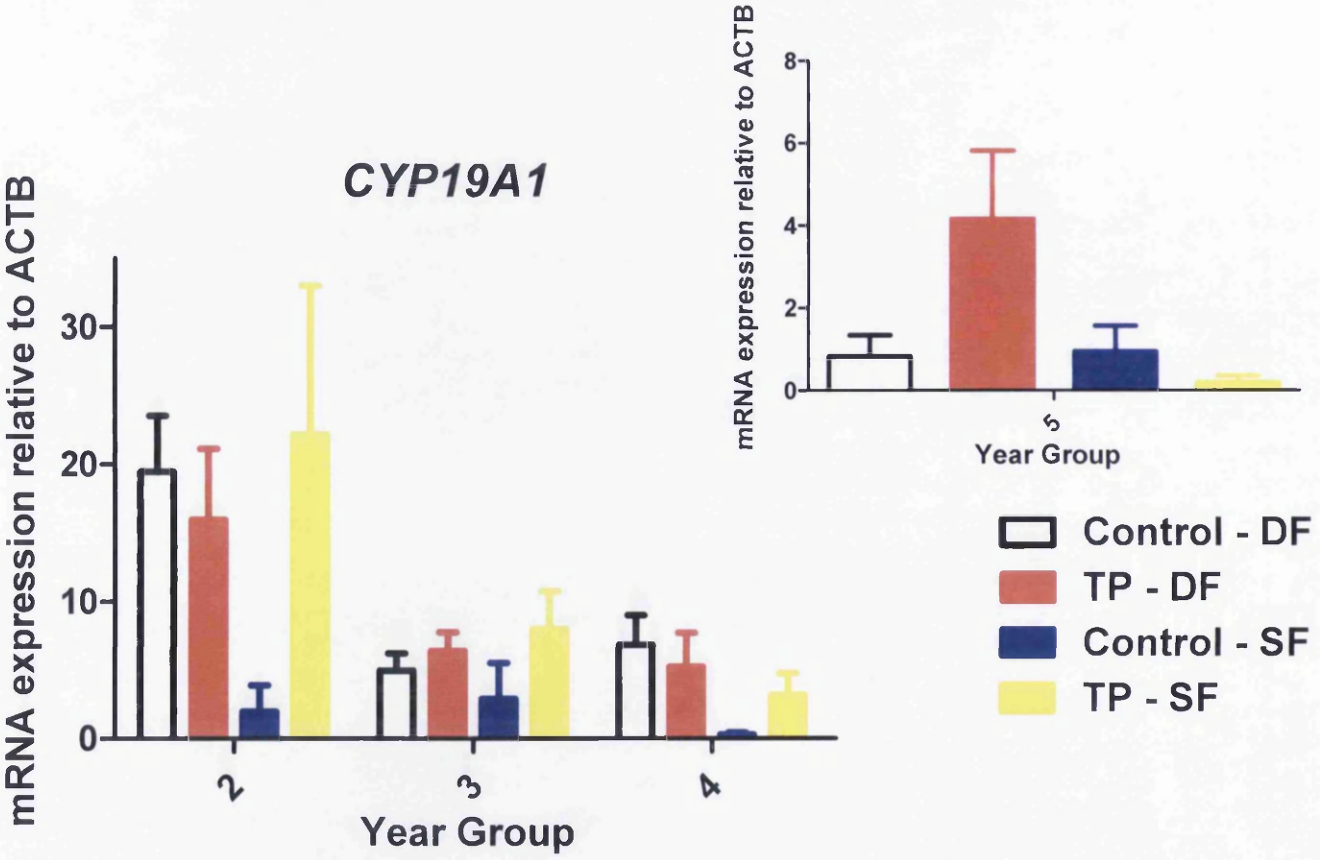
Figure 5.3 Mean *FSHR* exon 9/10 mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9 and Group 5 – C = 7; SF = 6 – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5 and Group 5 – C = 4; TP = 4). *ACTB* = β-Actin.

Expression of mRNA encoding *CYP19A1* and *HSD3B1* in granulosa cells of DF and SF.

Quantification of mRNA species showed that *CYP19A1* expression in granulosa cells of DF and SF was similar in TP-treated ewes compared to controls (Figure 5.4). Granulosa cell *CYP19A1* expression was reduced in granulosa cells from follicles classified as dominant compared to those classified as subordinate (Figure 5.6). Differences in the expression of *CYP19A1* mRNA between years was apparent, with expression in year group 3 significantly lower to all other year groups. In granulosa cells of DF and SF, *HSD3B1* was consistently expressed at a much higher level in TP-treated ewes compared to controls (Figure 5.5). *HSD3B1* mRNA levels in granulosa cells from DF follicles were reduced compared to those detected in SF follicles (Figure 5.6). Year group had a significant effect on granulosa cell *HSD3B1* expression, with levels in year group 5 being higher than in those observed in year groups 3 and 4. Analysis tables for *CYP19A1* and *HSD3B1* mRNA granulosa cell expression within DF and SF, which list the P values determined for each factor included within the statistical model, are shown in Figures 5.4 and 5.5, respectively.

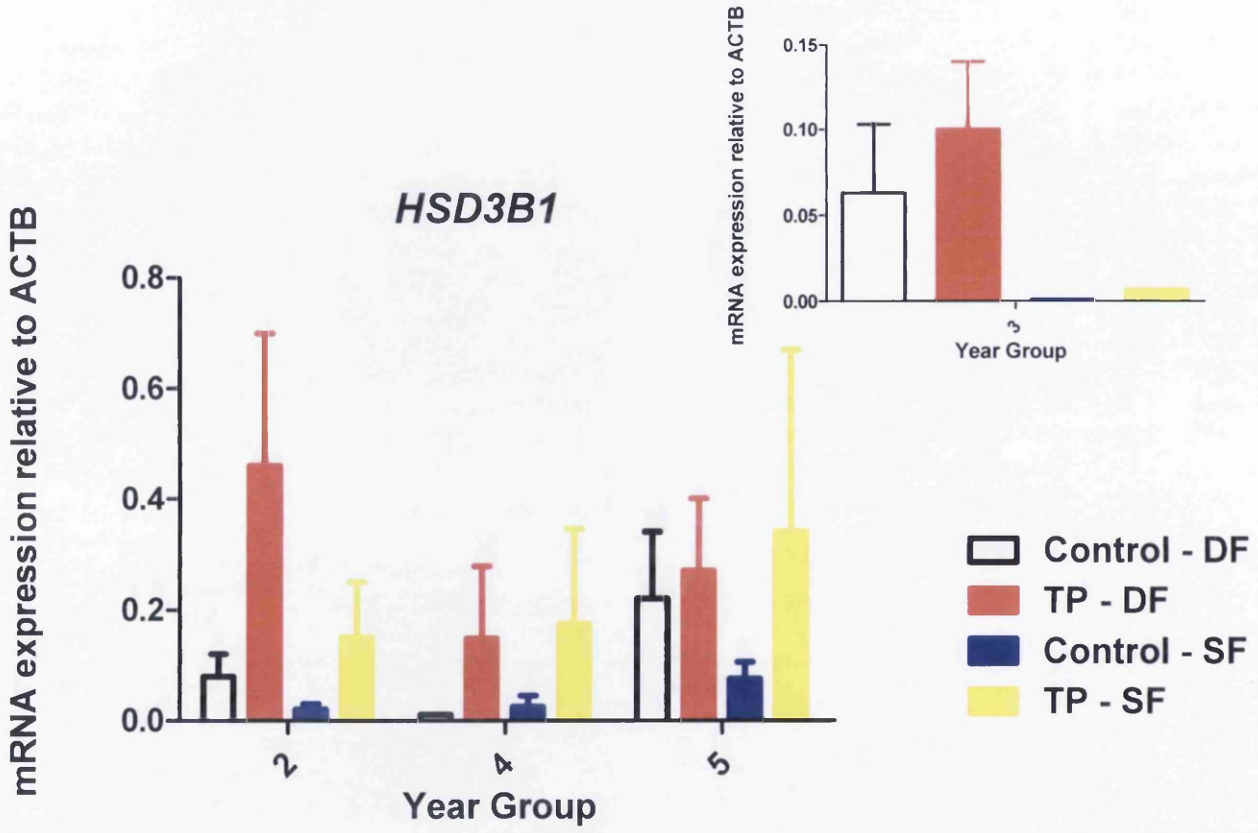
FSH dependent cell differentiation - Expression of mRNA encoding *INHA*, *INHBA* and *FST* in granulosa cells of DF and SF.

Quantification of mRNA species showed that *INHA, INHBA* and *FST* expression in granulosa cells of DF and SF were similar in TP-treated ewes compared to controls (Figures 5.7, 5.8 and 5.9, respectively). There was a tendency for the ewe year group to influence granulosa cell *INHA* mRNA expression, with levels in year group 3 higher than those within year group 2. While both GLM and GLS analysis showed that *FST* mRNA levels were increased in granulosa cells from DF follicles compared with those detected in SF follicles (Figure 5.13), only GLS analysis showed that SF *INHA* mRNA levels tended to be ($P < 0.1$) and SF *INHBA* mRNA expression was significantly higher in DF compared to SF granulosa cells (Figure 5.13). Analysis tables for *INHA*, *INHBA* and *FST* mRNA granulosa cell expression within DF and SF, which list the P values determined for each factor included within the statistical model, are shown in Figures 5.7, 5.8 and 5.9 respectively.



Factor	GLM P value
Year	0.000
Prenatal Treatment	0.174
Follicle Classification	0.001
Prenatal Treatment*Follicle Classification	0.475

Figure 5.4 Mean *CYP19A1* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3, 4 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 7; TP = 6 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 4; TP = 4 follicles). *ACTB* = β-Actin. Note that the vertical scale bar for year group 5 is different than that for years 2, 3 and 4.



Factor	GLM P value
Year	0.004
Prenatal Treatment	0.044
Follicle Classification	0.018
Prenatal Treatment*Follicle Classification	0.948

Figure 5.5 Mean *HSD3B1* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3, 4 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 7; TP = 6 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 4; TP = 4 follicles). *ACTB* = β-Actin. Note that the vertical scale bar for year group 3 is different from that for year groups 2, 4 and 5.

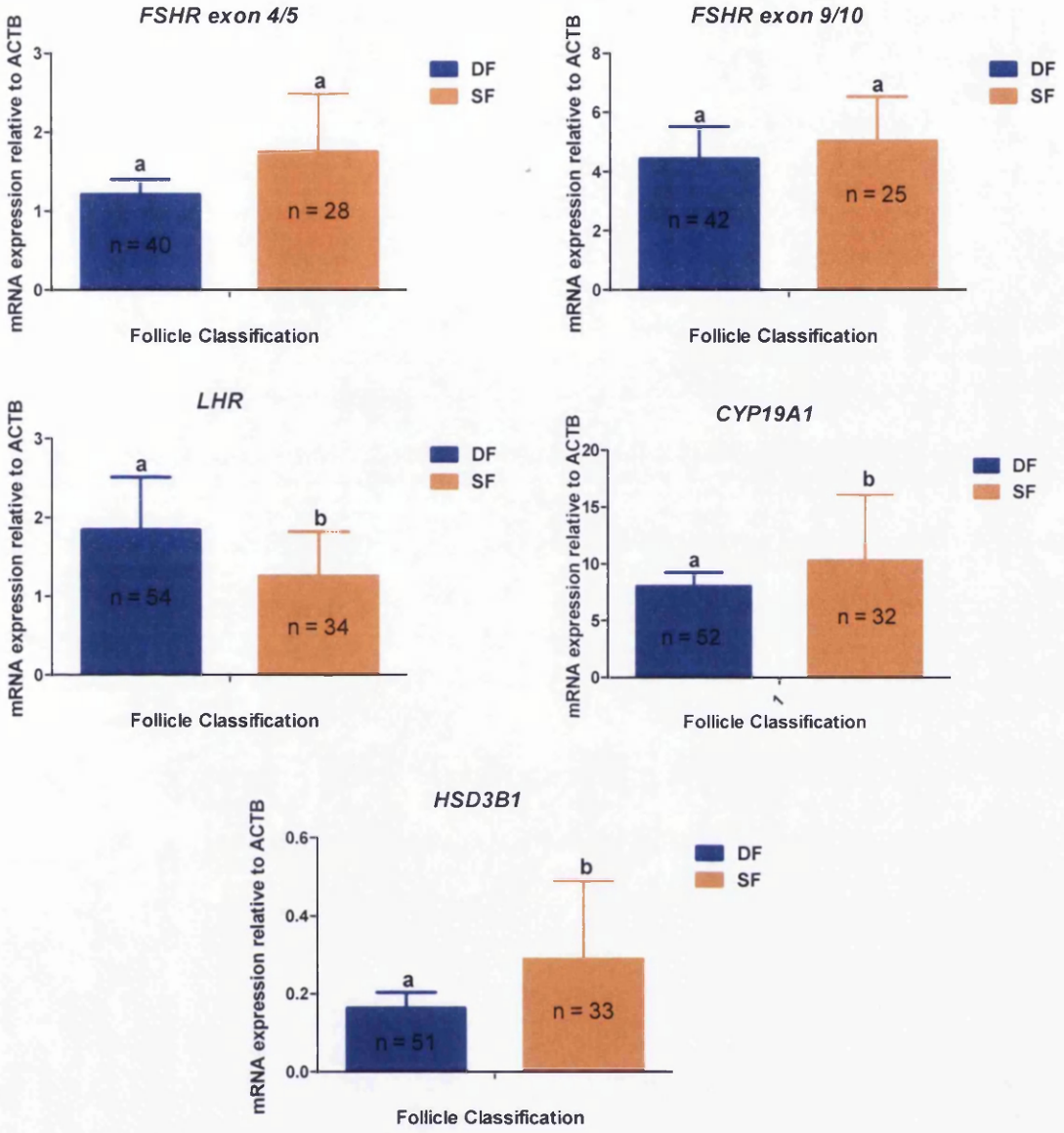
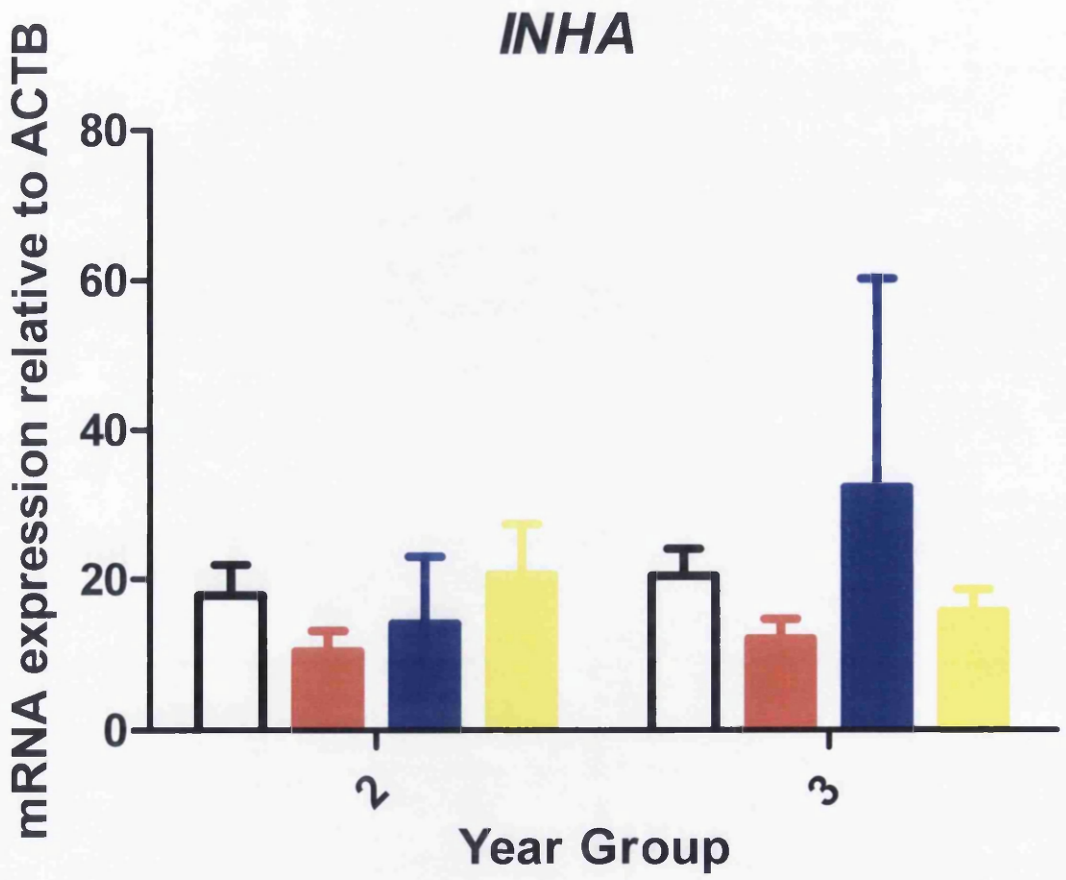
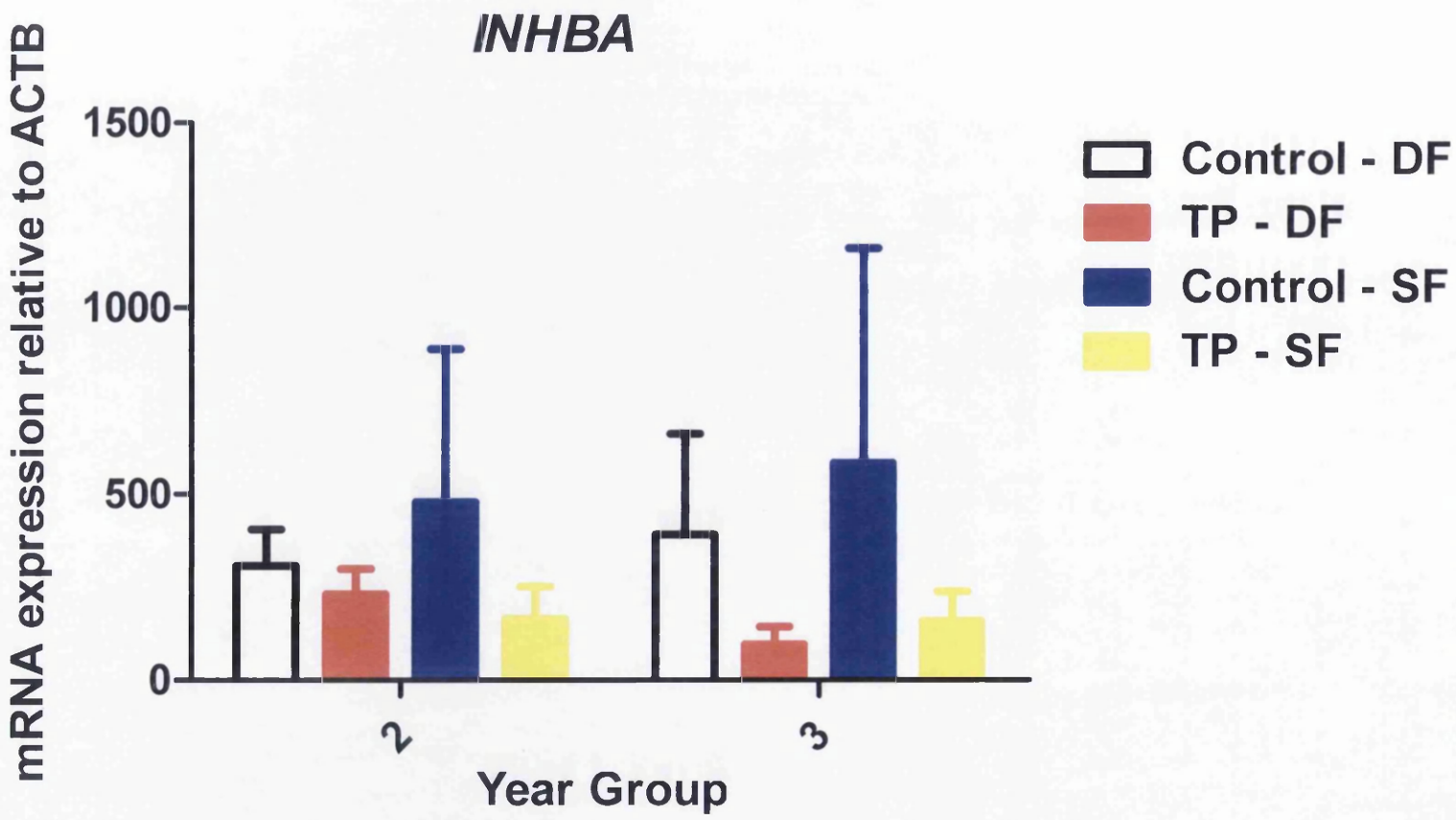


Figure 5.6 Mean mRNA expression in granulosa cells from dominant (DF) and subordinate (SF) follicles for the follicle differentiation markers follicle stimulating hormone receptor (*FSHR*), luteinising hormone receptor (*LHR*)) and steroidogenic enzymes (aromatase (*CYP19A1*) and 3-beta hydroxysteroid dehydrogenase (*HSD3B1*)) investigated in the study. Figure is based on combined data from the 4 year groups studied and combined across the two treatment groups. Values presented are mean ± SEM. *ACTB* = β-Actin [a,b] Within a follicle classification, means without a common superscript differ, when GLM analysis was used ($P < 0.05$).



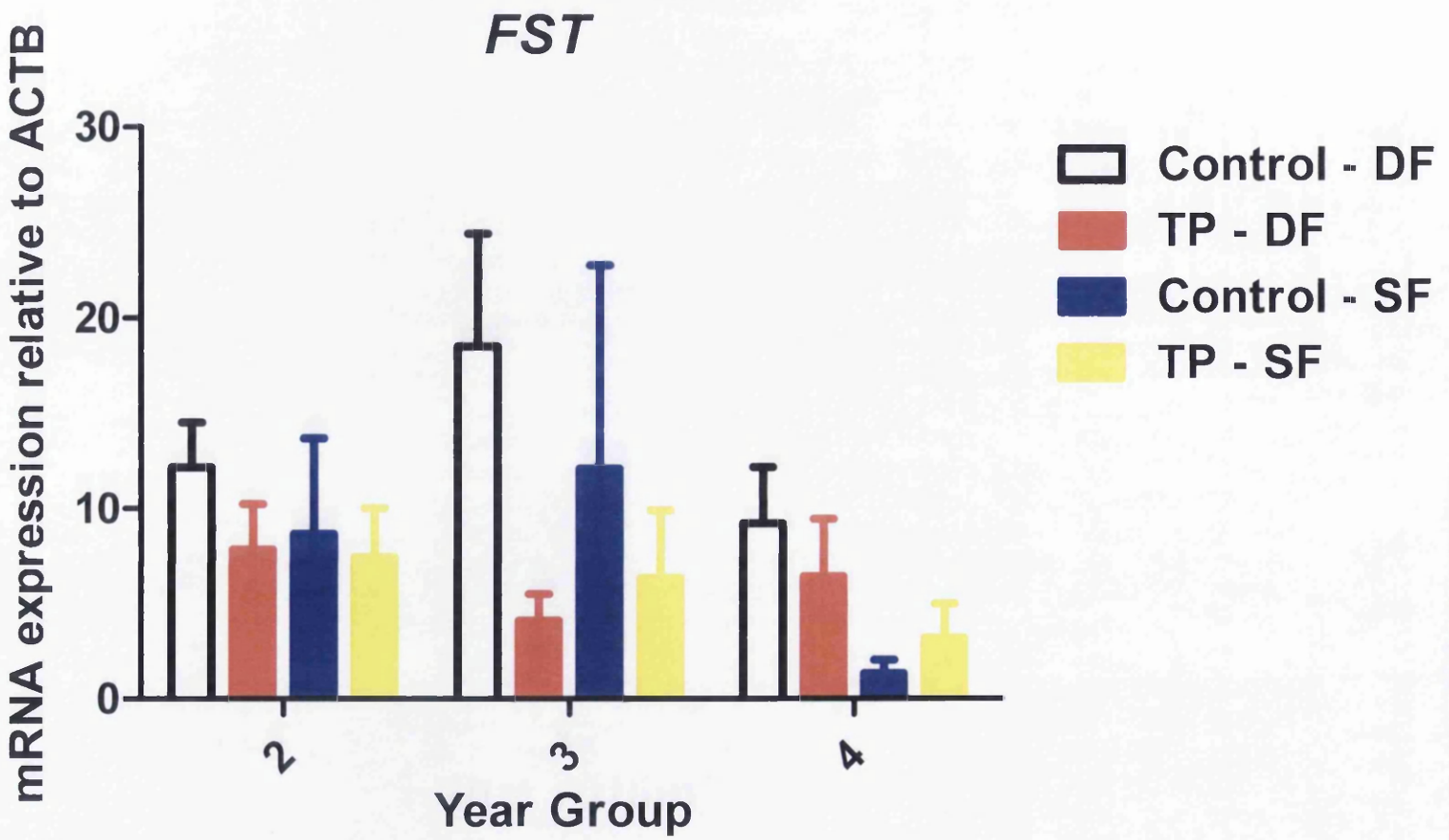
Factor	GLM P value (GLS)
Year	0.077
Prenatal Treatment	0.654
Follicle Classification	0.133 (0.078)
Prenatal Treatment*Follicle Classification	0.163

Figure 5.7 Mean *INHA* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2 and 3. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6 and Group 3 – C = 7; TP = 9 follicles – SF = Group 2 – C = 5; TP = 4 and Group 3 – C = 3; TP = 3 follicles). *ACTB* = β-Actin.



Factor	GLM P value (GLS)
Year	0.497
Prenatal Treatment	0.898
Follicle Classification	0.089 (0.050)
Prenatal Treatment*Follicle Classification	0.452

Figure 5.8 Mean *INHBA* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2 and 3. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6 and Group 3 – C = 7; TP = 9 follicles – SF = Group 2 – C = 5; TP = 4 and Group 3 – C = 3; TP = 4 follicles). *ACTB* = β-Actin.



Factor	GLM P value
Year	0.434
Prenatal Treatment	0.312
Follicle Classification	0.007
Prenatal Treatment*Follicle Classification	0.353

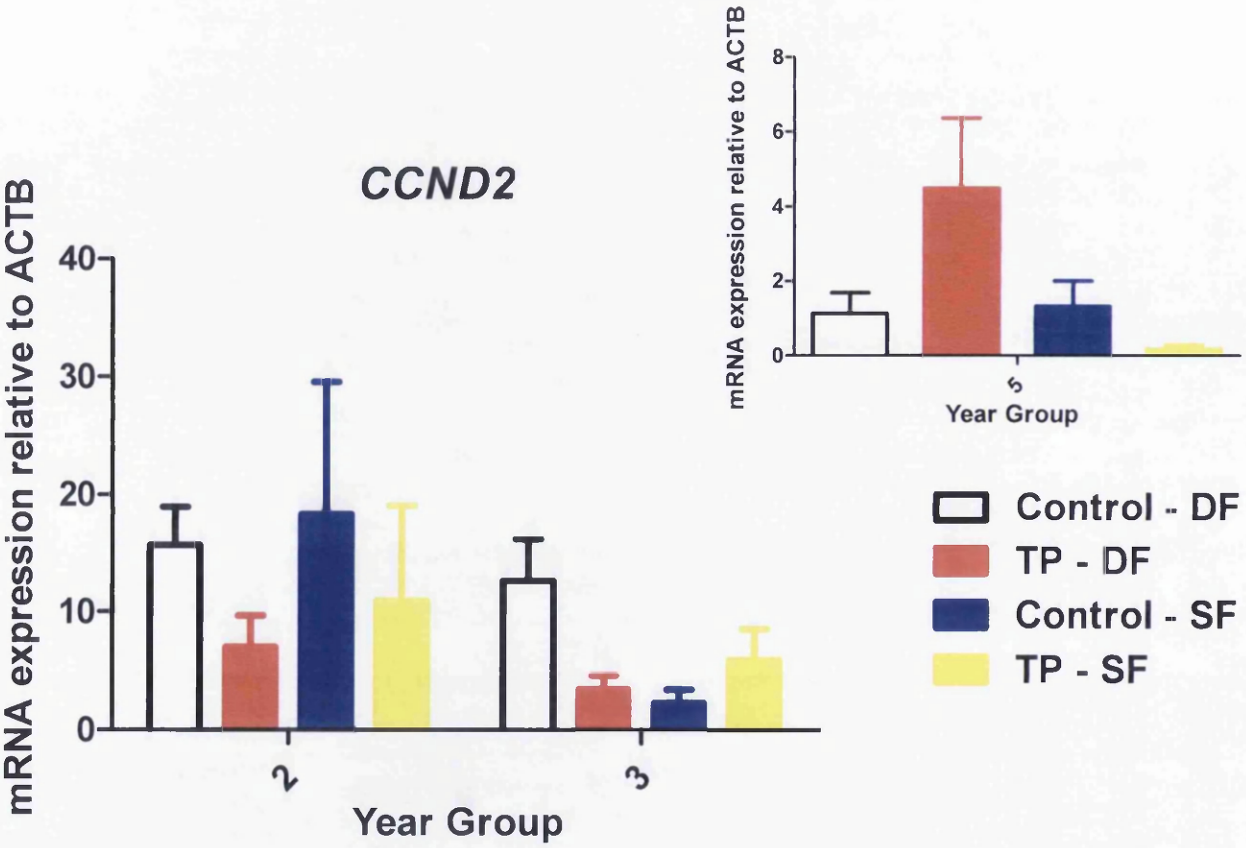
Figure 5.9 Mean *FST* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3 and 4. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9 and Group 4 – C = 7; TP = 5 follicles – SF = Group 2 – C = 5; TP = 4: Group 3 – C = 3; TP = 4 and Group 4 – C = 5; TP = 4 follicles). *ACTB* = β-Actin.

Cell proliferation and differentiation - Expression of mRNA encoding *CCND2, BGCAN* and *MIF* in granulosa cells of DF and SF.

Quantification of mRNA species showed that *CCND2* expression in granulosa cells of DF and SF was similar between TP-treated ewes and controls (Figure 5.10). Granulosa cell *CCND2* mRNA levels were reduced in year group 5 compared to year groups 2 and 3. Only GLS analysis determined that SF *CCND2* mRNA levels in granulosa cells were increased compared to transcript levels found in DF (Figure 5.13). Analysis tables for *CCND2*, *MIF* and *BGCAN* mRNA granulosa cell expression within DF and SF, which list the P values determined for each factor included within the statistical model, are shown in Figures 5.10-5.12, respectively. Quantification of mRNA species showed *MIF* expression in granulosa cells of DF and SF to be similar between TP-treated and control ewes (Figure 5.11). Only GLS analysis showed that granulosa cell *MIF* mRNA levels in DF tended ($P = 0.06$) to be higher compared with those detected in SF (Figure 5.13). Granulosa cell *MIF* mRNA levels were reduced in year group 5 compared to year groups 2, 3 and 4, while expression levels were increased in year group 4 compared to year group 3. Prenatal androgenisation by TP had no effect on the granulosa cell expression of *BGCAN* mRNA in DF and SF recovered from each ewe (Figure 5.12). Equally, *BGCAN* mRNA levels in granulosa cells from DF were similar to those detected in SF (Figure 5.13). Granulosa cell *BGCAN* mRNA levels were reduced in year group 2 compared to year group 3.

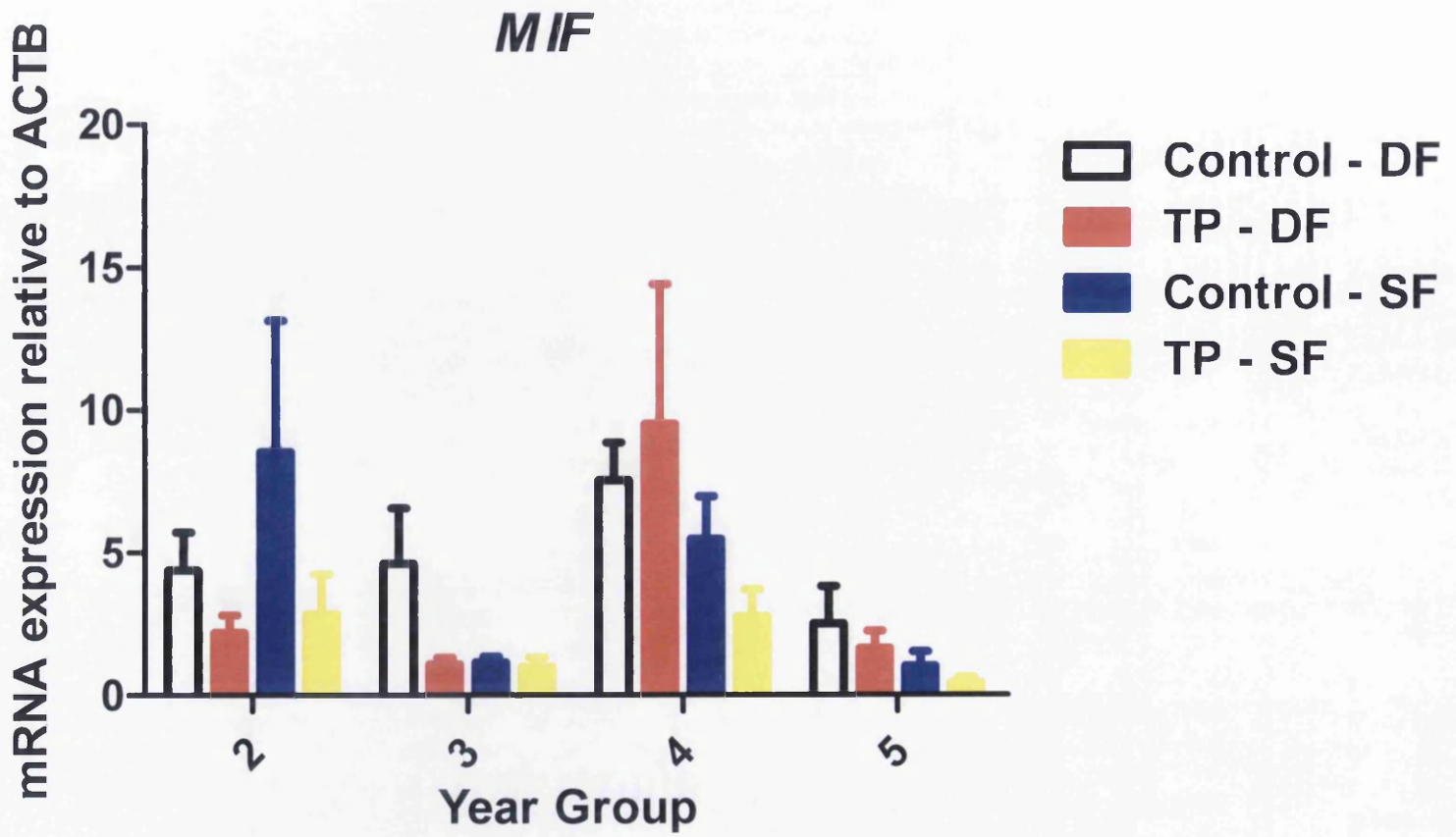
Cell apoptosis - Expression of mRNA encoding *MCL-1, CASP3* and *BAX* in granulosa cells of DF and SF.

Granulosa cell *MCL-1* mRNA expression levels in DF and SF were similar between treatment groups (Figure 5.14). There was also no difference in granulosa cell *MCL-1* expression between DF and SF (Figure 5.17), while expression levels were reduced in year group 3 compared to year group 2. Expression levels of *CASP3* mRNA in the granulosa cells of DF and SF recovered from each ewe were unchanged when ewes were prenatally treated with TP (Figure 5.15). *CASP3* mRNA levels in granulosa cells from DF follicles were similar to those detected in SF follicles (Figure 5.17). Granulosa cell *CASP3* mRNA levels were reduced in year group 2 compared to year groups 3 and 5. Prenatal androgenisation by TP had no effect on the expression of *BAX* mRNA in the granulosa cells of DF and SF recovered from each ewe (Figure 5.16). *BAX* mRNA levels in granulosa cells from DF were similar to those detected in SF (Figure 5.17). Granulosa cell *BAX* mRNA levels were increased in year group 2 compared to year group 3. Analysis tables for *MCL-1, CASP3* and *BAX* mRNA granulosa cell expression within DF and SF, which list the P values determined for each factor included within the statistical model, are shown in Figures 5.14, 5.15 and 5.16, respectively.



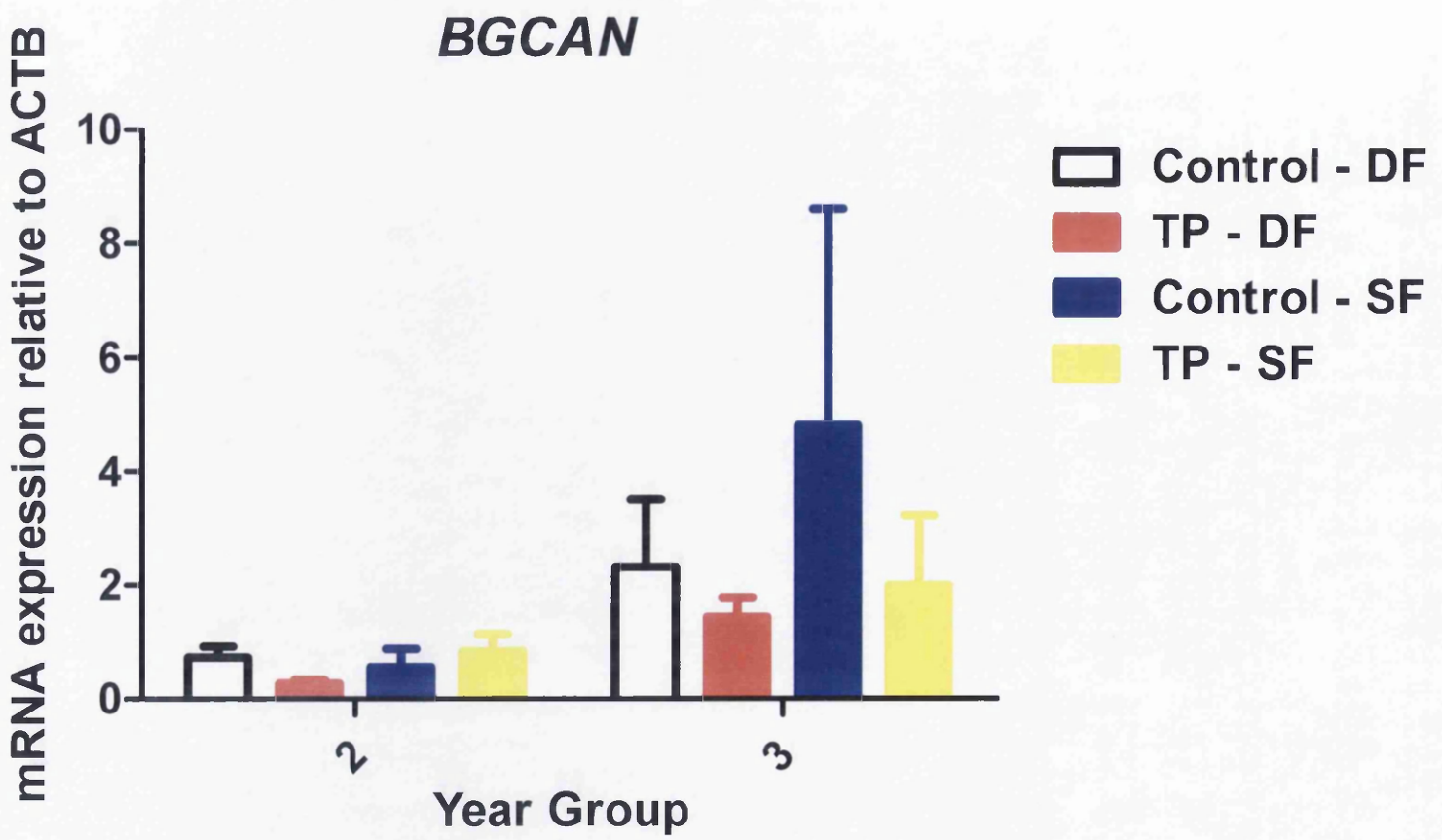
Factor	GLM P value (GLS)
Year	0.000
Prenatal Treatment	0.340
Follicle Classification	0.239 (0.047)
Prenatal Treatment*Follicle Classification	0.171

Figure 5.10 Mean *CCND2* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9 and Group 5 – C = 7; TP = 6 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5 and Group 5 – C = 4; TP = 4 follicles). *ACTB* = β-Actin. Note that the vertical scale bar for year group 5 is different from than for year groups 2 and 3.



Factor	GLM P value (GLS)
Year	0.000
Prenatal Treatment	0.437
Follicle Classification	0.361 (0.056)
Prenatal Treatment*Follicle Classification	0.326

Figure 5.11 Mean *MIF* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3, 4 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9: Group 4 – C = 7; TP = 5 and Group 5 – C = 7; TP = 6 follicles – SF = Group 2 – C = 5; TP = 5: Group 3 – C = 3; TP = 5: Group 4 – C = 6; TP = 4 and Group 5 – C = 4; TP = 4 follicles). *ACTB* = β-Actin.



Factor	GLM P value
Year	0.014
Prenatal Treatment	0.411
Follicle Classification	0.655
Prenatal Treatment*Follicle Classification	0.903

Figure 5.12 Mean *BGCAN* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2 and 3. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6 and Group 3 – C = 7; TP = 9 follicles – SF = Group 2 – C = 5; TP = 4 and Group 3 – C = 3; TP = 4 follicles). *ACTB* = β-Actin.

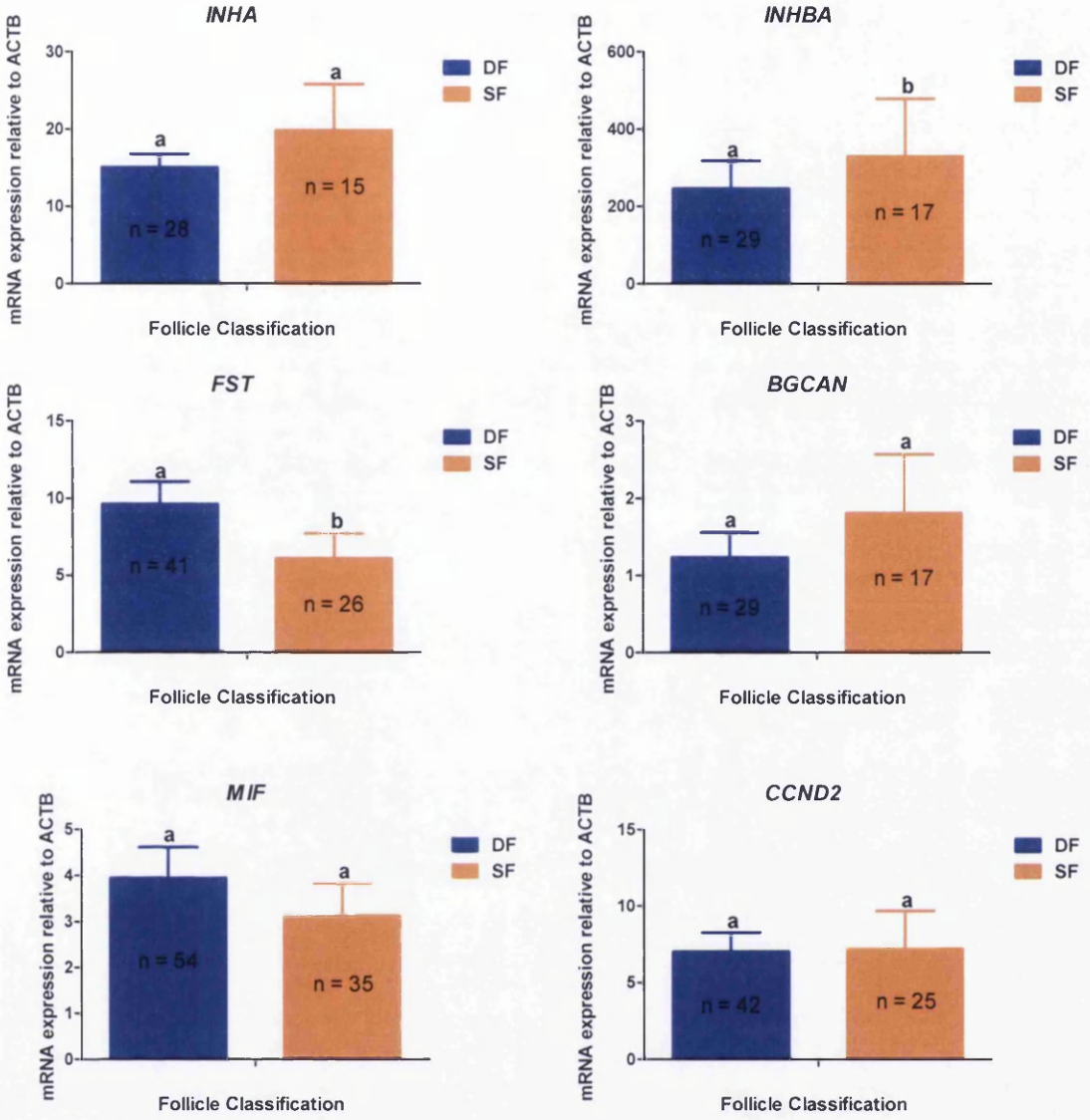
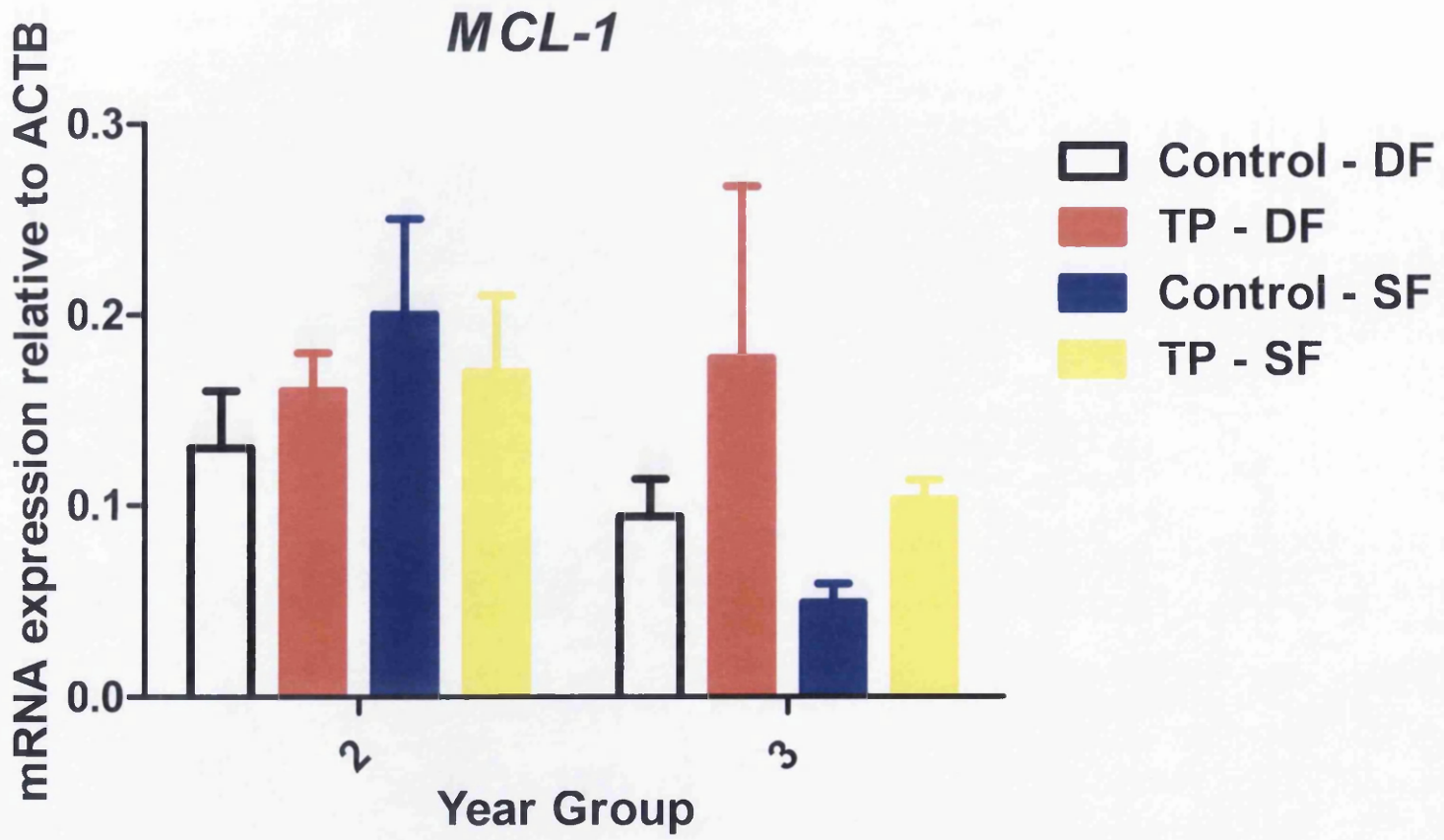
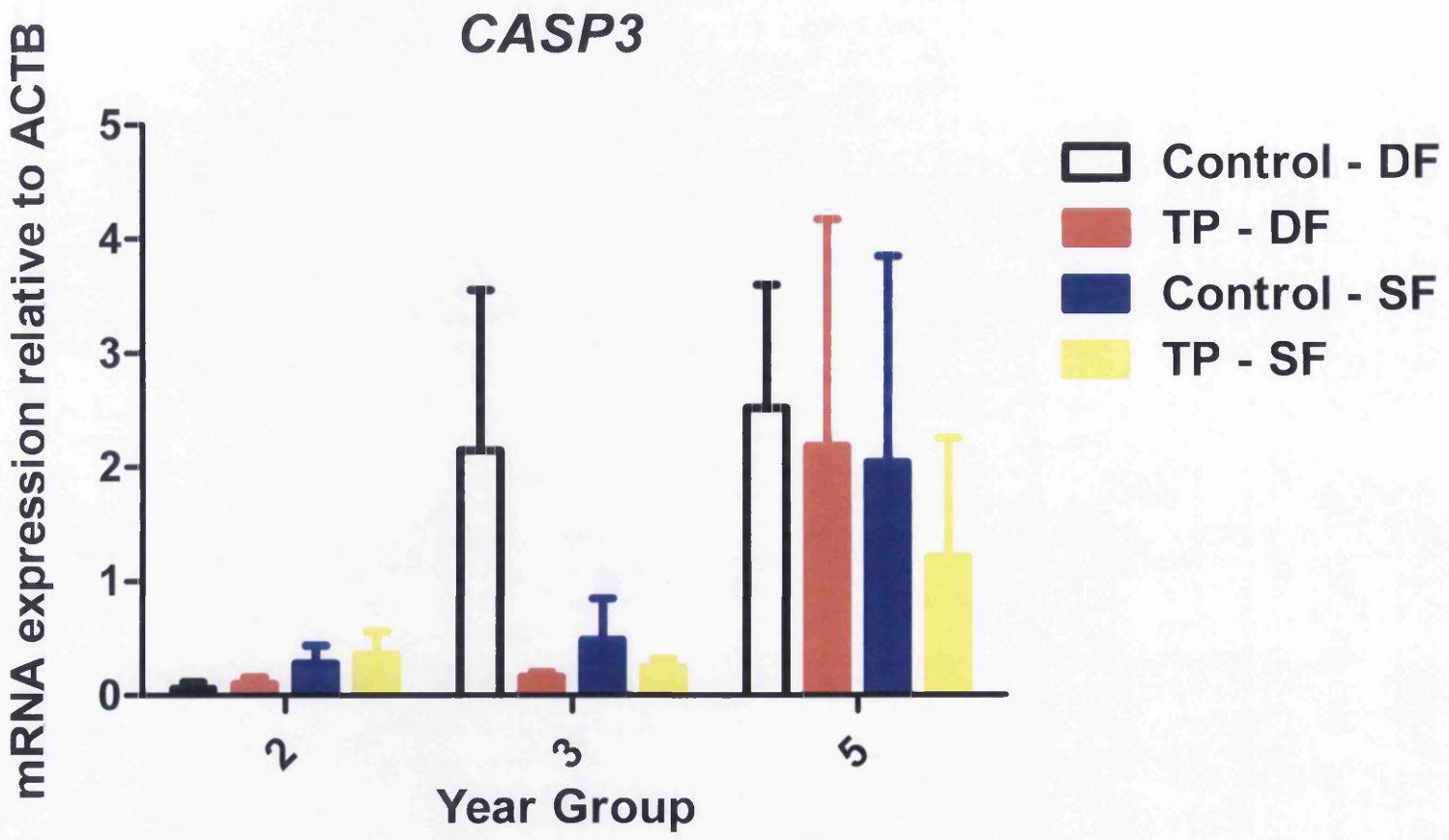


Figure 5.13 Mean mRNA expression in granulosa cells from dominant (DF) and subordinate (SF) follicles for the FSH dependent follicle differentiation markers (inhibin subunit alpha (*INHA*), inhibin subunit beta A (*INHBA*) and follistatin (*FST*)), follicle differentiation markers (betaglycan (*BGCAN*) and macrophage migration inhibitory factor (*MIF*)) and the follicle proliferation marker cyclin D2 (*CCND2*) investigated in the study. Figure is based on combined data from the 4 year groups studied and combined across the two prenatal treatment groups. Values presented are mean ± SEM. *ACTB* = β-Actin [a,b] Within a follicle classification, means without a common superscript differ, when GLM analysis was used ($P < 0.05$).



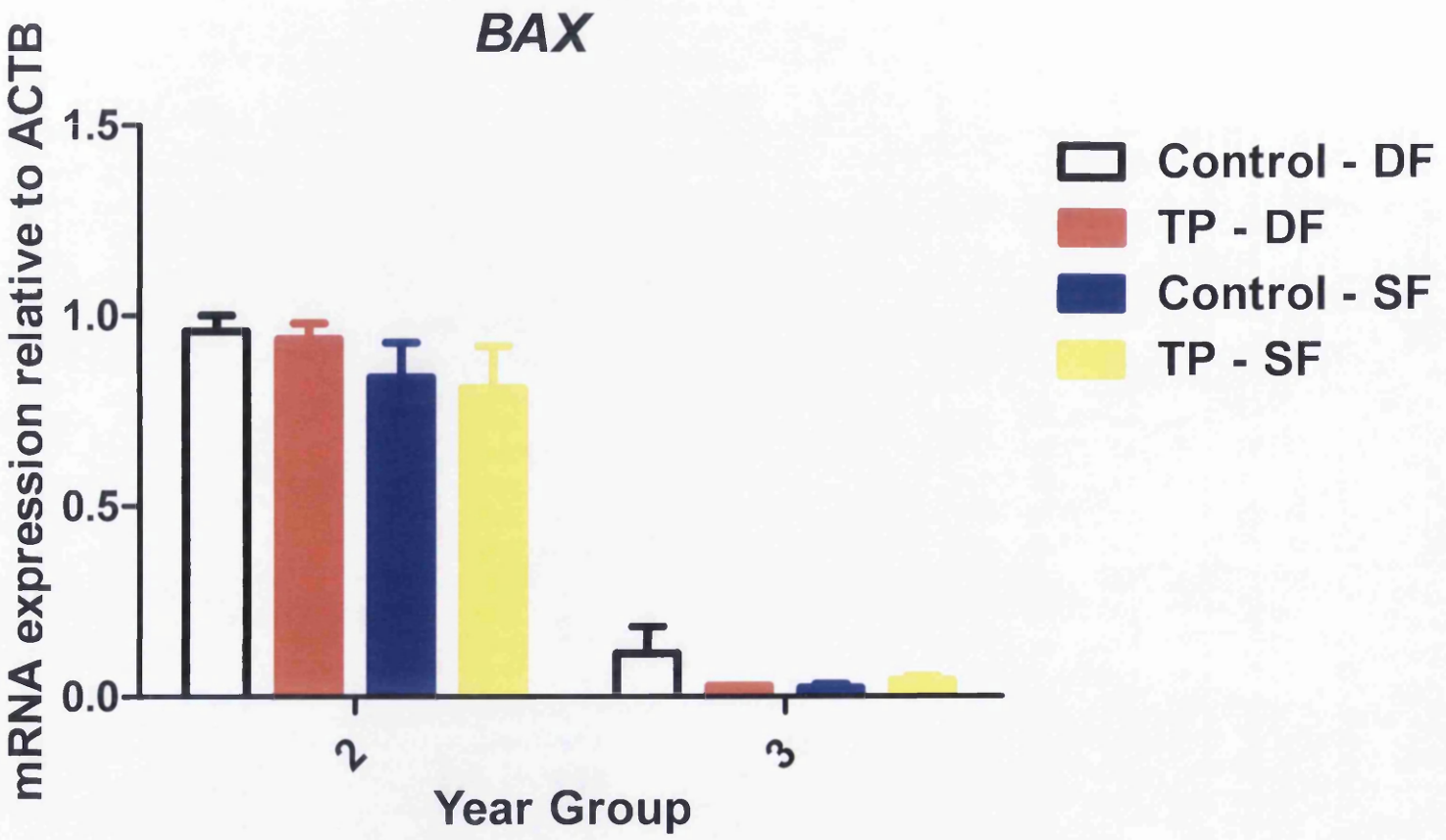
Factor	GLM P value
Year	0.023
Prenatal Treatment	0.432
Follicle Classification	0.576
Prenatal Treatment*Follicle Classification	0.679

Figure 5.14 Mean *MCL-1* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2 and 3. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6 and Group 3 – C = 7; TP = 9 follicles – SF = Group 2 – C = 5; TP = 4 and Group 3 – C = 3; TP = 4 follicles). *ACTB* = β-Actin



Factor	GLM P value
Year	0.000
Prenatal Treatment	0.376
Follicle Classification	0.550
Prenatal Treatment*Follicle Classification	0.316

Figure 5.15 Mean *CASP3* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2, 3 and 5. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6: Group 3 – C = 7; TP = 9 and Group 5 – C = 7; TP = 6 follicles – SF = Group 2 – C = 5; TP = 4; Group 3 – C = 3; TP = 4 and Group 5 – C = 4; TP = 4 follicles). *ACTB* = β-Actin.



Factor	GLM P value
Year	0.000
Prenatal Treatment	0.767
Follicle Classification	0.357
Prenatal Treatment*Follicle Classification	0.316

Figure 5.16 Mean *BAX* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control and prenatally androgenised ewes in year groups 2 and 3. Values presented are mean ± SEM. (DF = Group 2 – C = 7; TP = 6 and Group 3 – C = 7; TP = 9 follicles – SF = Group 2 – C = 5; TP = 4 and Group 3 – C = 3; TP = 4 follicles). *ACTB* = β-Actin.

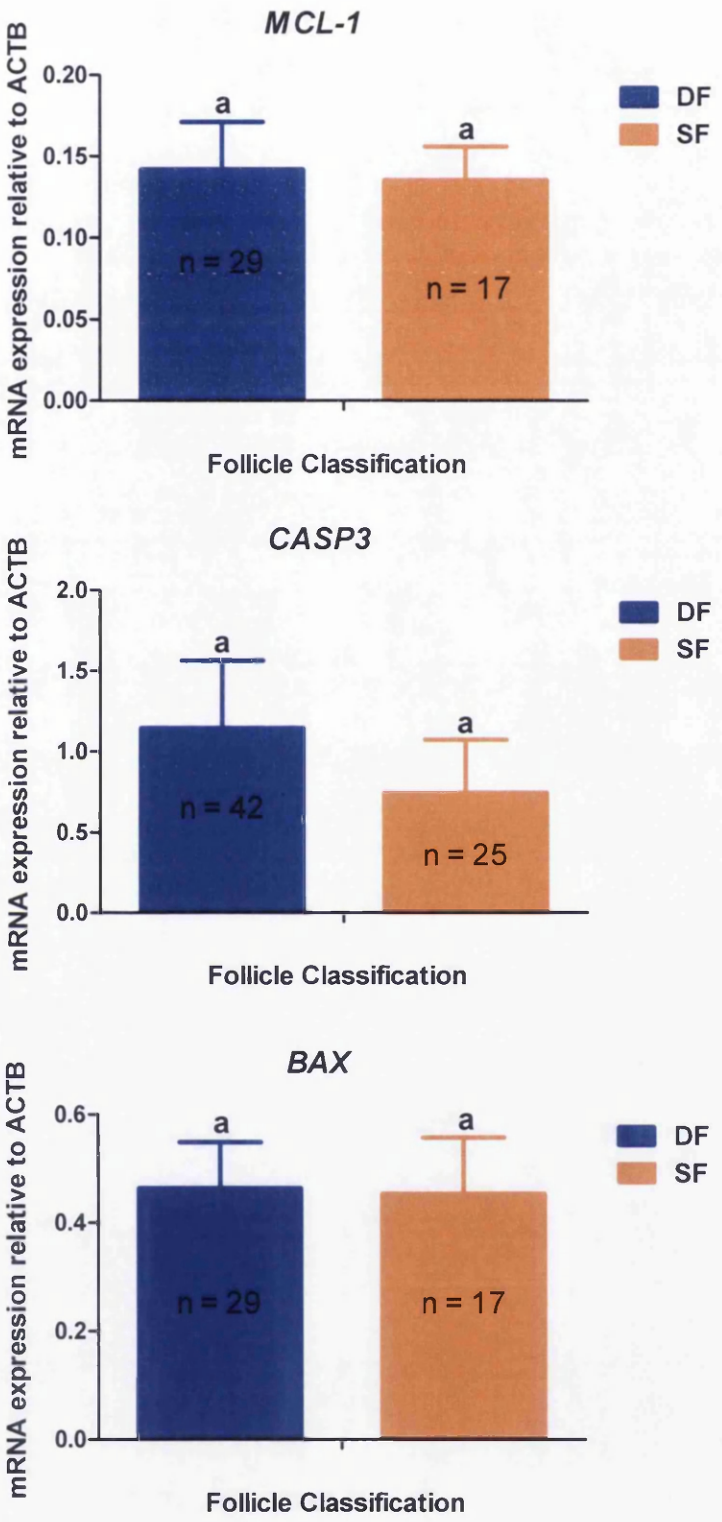


Figure 5.17 Mean mRNA expression in granulosa cells from dominant (DF) and subordinate (SF) follicles for the anti-apoptotic myeloid cell leukaemia factor 1 (*MCL-1*) and the pro-apoptotic marker genes caspase 3 (CASP3) and BCL-2 associated protein X (*BAX*) investigated in the study. Figure is based on combined data from the 4 year groups studied and combined across the two treatment groups. Values presented are mean ± SEM. *ACTB* = β-Actin ^{a,b} Within a follicle classification, means without a common superscript differ, when GLM analysis was used ($P < 0.05$).

5.4 Discussion

This study is the first to have identified changes in granulosa cell transcripts in DF and SF as a result of prenatal programming by excess TP. The approach used within this study differs from that of previous investigations into the effects of programming through prenatal androgen excess in that we have determined molecular changes within granulosa cells recovered from individually identified and characterised DF and SF, rather than investigation into the endocrine environment (Robinson *et al.*, 1999; Sarma *et al.*, 2005; Savabieasfahani *et al.*, 2005), or antral follicle persistence, and histological studies into antral follicle numbers or follicular gene expression (Steckler *et al.*, 2005; Manikkam *et al.*, 2006; Steckler *et al.*, 2007a; Smith *et al.*, 2008).

Prenatal androgenisation by TP increased mRNA expression for *LHR* and *HSD3B1* and reduced mRNA expression for *FSHR* in follicular granulosa cells of the largest follicles recovered (DF and SF) from each ewe over several years. Prenatal androgenisation by TP had no consistent effect on the mRNA expression for granulosa cell genes regulating oestradiol production (*CYP19A1*), FSH-dependent cell differentiation (*INHA*, *INHBA* and *FST*) and proliferation (*CCND2*), differentiation (*BGCAN* & *MIF*), and apoptosis (*MCL-1*, *MIF*, *CASP3* & *BAX*). The implications of these findings on follicle development within prenatally androgenised ewes are discussed below.

It is again pertinent to highlight that, as in **Chapter 3**, significant differences between year groups (despite ewes being of the same age) were attained for several genes. Possible reasons for these differences were discussed in the third chapter. In particular, year groups 2 and 5 show most differences to the other year groups and are characterised by an almost opposing (in terms of predicted functions of proteins) transcript profile. Year group 2 granulosa cells express more mRNA for *LHR*, *CYP19A1*, *CCND2*, *MCL-1* but also *BAX*, and less mRNA for *INHA*, *BGCAN* and *CASP3*. In contrast, year group 5 granulosa cells express more mRNA for *HSD3B1* and *CASP3*, but less mRNA for *LHR*, *CYP19A1*, *CCND2* and *MIF*. This translated into only a few differences in follicular fluid oestradiol to progesterone ratio (lower in year group 5 compared to year groups 3 and 4) between year groups, but not into differences in follicle growth between years.

In addition, several differences in transcript levels were detected between dominant and subordinate follicles. Within DF, *LHR*, *FST* and *MIF* (GLS only) mRNA gene expression was higher compared to SF, while *FSHR* exon4/5 (GLS only), *HSD3B1*, *CYP19A1*, *INHA*

(GLS only), *INHBA* (GLS only) and *CCND2* (GLS only) mRNA gene expression was reduced in DF compared to SF.

Increased mRNA expression of *LHR* in granulosa cells of DF and SF from TP-treated ewes suggests increased ability to respond to peripheral concentrations of LH, as previous studies in the bovine have shown that mRNA and protein binding changes go hand in hand (Ireland & Roche, 1983). Hypersecretion of LH in androgenised Dorset (West *et al.*, 2001) and Suffolk (Sarma *et al.*, 2005) ewes, together with the proposed increased responsiveness to LH implies that increased follicular steroidogenesis and enhanced growth may be driven by the actions of LH. Increased LH pulsatility stimulates follicular growth and oestradiol production in follicles with increasing LH responsiveness as seen in the follicular phase of the cycle (Lucy, 2007), and in dominant follicles made persistent by extending a frequent LH pulse profile while inhibiting any surges (Bridges & Fortune, 2003). It is also well known that, within granulosa cells of large antral follicles, LH stimulates the synthesis of progesterone (Denning-Kendall & Wathes, 1994). Thus, LH may be the gonadotrophin that induces abnormally large follicle development combined with high oestradiol and progesterone secretion in androgenised ewes. A reduction in *FSHR* mRNA expression provides further evidence that increased follicle steroidogenesis and growth is driven more by the actions of LH than FSH. This avenue should be explored further in order to provide conclusive poof that the ovarian abnormalities of increased growth and steroidogenesis are as a result of the actions of LH. In a proposed future *in vivo* study, endogenous FSH and LH secretion would be inhibited in the long-term (by means of a GnRH antagonist or potent agonist) in control and prenatally androgenised ewes. Exogenous LH would then be administered in a manner that replicates the peripheral LH concentrations and episodic profiles seen in prenatally androgenised ewes (Sarma *et al.*, 2005). The resultant ovarian morphology and histology, and the follicular expression profiles of *LHR* and *FSHR* (real-time PCR), in addition to other genes found to be abnormally regulated, such as *HSD3B1* would then be investigated. A recent short-term study of GnRH antagonist treatment with gonadotrophin supplementation in the follicular phase was carried out in the prenatally androgenised Suffolk model (with a less severe phenotype), showing that persistent large follicles are still able to respond to different LH environments with atresia or ovulation (Steckler *et al.*, 2008). This supports the concept of high LH responsiveness in large growing follicles from prenatally androgenised ewes.

Reduced mRNA expression of *FSHR* concomitant with an increase in *LHR* expression in granulosa cells of DF and SF from TP-treated ewes provides further evidence that prenatal

androgenisation by TP results in enhanced DF and SF follicle differentiation, as both these molecular characteristics are markers of continued dominant follicle development (Mihm *et al.*, 2006). Such enhanced differentiation could represent a degree of premature luteinisation of granulosa cells within DF and SF from androgenised ewes in the absence of an LH surge. However, in luteinising follicles *in vivo* and *in vitro* oestradiol secretion is abandoned in favour of progesterone secretion (Auletta & Flint, 1988), which is clearly not the case in follicles from this androgenised ewe model. Further study into whether increased granulosa cell mRNA *LHR* and reduced *FSHR* expression results in a differential response to the gonadotrophins *in vitro* is necessary.

Prenatal androgenisation resulting in a reduction in granulosa cell *FSHR* expression within DF and SF is in stark contrast to results obtained in women affected by PCOS (Catteau-Jonard *et al.*, 2008). Here, granulosa cell *FSHR* expression was significantly higher in small (8-13 mm) and large (17-22 mm) follicles recovered from PCOS patients undergoing controlled ovarian hyperstimulation during a cycle for subsequent *in vitro* fertilisation (Catteau-Jonard *et al.*, 2008). Differences between the two studies could be as a result of the controlled ovarian hyperstimulation resulting in a much elevated peripheral FSH environment that does not replicate the hormonal environment typically present in prenatally androgenised ewes (McNatty *et al.*, 1985; Fry & Driancourt, 1996; Manikkam *et al.*, 2008).

Reduced mRNA expression for *FSHR*, increased mRNA expression for *LHR* in granulosa cells and increased oestradiol and progesterone follicular fluid concentrations in both DF and SF from prenatally androgenised ewes seen in **Chapter 3** implies that the follicle hierarchy is lost and this, to a certain extent, abolishes follicle selection. However, differences seen between DF and SF in *LHR* and *FSHR* mRNA expression were clearly present in both treatments (see below), as no interaction between prenatal treatment and follicle classification in granulosa cell mRNA expression levels of the three gonadotrophin receptors studied (*LHR* and the two *FSHR* transcripts) was present. Thus, this finding implies that a follicle hierarchy is still maintained in terms of LH responsiveness in the largest follicles, mirroring the results for intrafollicular oestradiol, in prenatally androgenised ewes.

Elevated granulosa cell *LHR* mRNA expression in follicles classified as DF compared to SF provided additional validation of the follicle classification criteria used in the study, as *LHR* expression is upregulated within oestrogenic DF in the bovine (Mihm *et al.*, 2006;

Ryan *et al.*, 2007). Further evidence for validation of our follicle classification criteria was obtained by the observation that granulosa cell *FSHR* mRNA expression tended to be downregulated (GLS analysis only) in follicles classified as DF in comparison to SF, which parallels that seen in other ruminant studies (Evans *et al.*, 2004; Mihm *et al.*, 2008). Conversely, no such decrease in granulosa cell *FSHR* exon 9/10 mRNA, the signalling domain of the receptor, was determined in DF compared to SF, which appears to point towards similar functional FSHR transcription in DF and SF in our study. Finally, *CYP19A1* expression was reduced in follicles classified as dominant compared to those classified as subordinate. While this does not support the oestradiol results obtained from follicular fluid analysis, it seems that the absolute high levels of CYP19A1 transcripts within granulosa cells of SF recovered from TP-treated ewes are responsible for this observation, yet no interaction between prenatal treatment and follicle classification was determined.

Prenatal androgenisation through TP resulted in increased mRNA expression levels of *HSD3B1* in granulosa cells of DF and SF. Increased progesterone production seen within DF and SF may, therefore, be largely as a result of increased granulosa cell *HSD3B1* expression, most likely as a result of increased stimulation by intracellular cAMP due to increased responsiveness to LH (Spicer *et al.*, 1993). Granulosa cell *HSD3B1* expression within DF was reduced in comparison to SF. This result does not support our finding of equal progesterone production in the two largest follicles recovered or results from ovine follicle wave studies showing that intrafollicular progesterone is increased in DF compared to SF (Evans & Martin, 2000). One possible explanation for this finding is that the control SF isolated in our study may have included older dominant follicles with higher HSD3B1 transcription. Also, increased *HSD3B1* enzyme transcription may compensate for the reduced LH responsiveness leading to similar progesterone production in SF. No significant prenatal treatment and follicle classification interaction was determined, thus prenatal TP treatment does not specifically program increased granulosa cell *HSD3B1* expression in SF. Further study into whether the increased follicular fluid progesterone within DF and SF is as a result of an inherent increase in granulosa cell progesterone production, or if the increase is driven by increased responsiveness to LH is required.

In the present study, mRNA expression levels for *CYP19A1* in granulosa cells of DF and SF were unchanged by prenatal androgenisation. This does not concur with our finding that androgenisation by TP resulted in increased follicular fluid concentrations of oestradiol in DF and SF (**Chapter 3**). Therefore, increased oestradiol production from DF and SF of

prenatally TP-treated ewes is not due to increased transcription of the aromatase gene. Increased oestradiol synthesis may still occur with maintained but not elevated aromatase transcription as the reverse is seen in cultured bovine dominant follicles, where no changes in granulosa cell aromatase activity are present, despite dominant follicles losing their oestrogen activity when they become atretic (Badinga *et al.*, 1992). Additionally, *CYP19A1* mRNA levels are unchanged when induced by FSH in growing bovine dominant follicles despite changes in follicular fluid oestradiol (Bao *et al.*, 1997). Thus, it appears that it is the translational or post-translational control of aromatase activity which is significant for aromatase function in follicles. Increased follicular fluid oestradiol concentrations in androgenised DF and SF may be as a result of increased precursor availability compared with controls, in combination with maintained high aromatase expression. Increased precursor concentrations (of testosterone and androstenedione) within theca cells for subsequent conversion to oestradiol should be investigated in TP-treated ewes, by both determining the mRNA expression and protein levels of the enzymes necessary to produce them, such as cytochrome P450 17α-hydroxylase (CYP17) and cytochrome P450 side chain cleavage (CYP11A) (Jakimiuk *et al.*, 2001; Amsterdam *et al.*, 2003). As expected, we did not observe increased precursor (testosterone) in follicular fluid of androgenised ewes, as the predicted high aromatase activity should prevent such accumulation.

Previous studies into granulosa cell expression of follicles less than 7 mm in diameter (an earlier stage of follicle development) revealed that *CYP19A1* levels were actually lowered in follicles recovered from PCOS patients (Jakimiuk *et al.*, 1998). However, as follicles do not progress much further in development than 8 mm in diameter in PCOS patients without FSH hyperstimulation (Forsdike *et al.*, 2007), we cannot directly compare *CYP19A1* expression results to those from this study where follicles are in the terminal stages of follicle development.

Given the increased steroidogenesis within DF and SF recovered from TP-treated ewes seen in **Chapter 3**, the relative expression levels of steroidogenic acute regulatory protein (StAR) should be investigated. The rate-limiting step in gonadal and adrenal steroidogenesis is the transfer of cholesterol from the relatively cholesterol rich outer mitochondrial membrane to the cholesterol-poor inner mitochondrial membrane (Strauss *et al.*, 1999; Miller, 2007). StAR mediates this rate-limiting step of steroidogenesis, delivering cholesterol to the inner mitochondrial membrane (Strauss *et al.*, 1999; Miller, 2007). Within bovine DF, any increase in StAR mRNA expression co-incides with an

accumulation of intrafollicular steroids (Nimz *et al.*, 2009). As a result of prenatal androgenisation by TP increasing steroidogenesis, we would propose that the expression of StAR is upregulated, thus increasing the availability of cholesterol within the inner mitochondrial membrane for subsequent conversion to pregnenolone by cytochrome P450 side chain cleavage (CYP11A).

Investigation into the granulosa cell mRNA expression profiles for the FSH dependent follicle differentiation marker genes *INHA*, *INHBA* and *FST* revealed no differences between treatment groups. These results support the hypothesis that the ovarian dysfunction seen is most likely as a result of LH action only and not FSH. Prenatal androgenisation by TP had also no effect on the granulosa cell transcription of the *BGCAN* gene which, if upregulated, would possibly have indicated enhanced inhibin action and diminished activin action (Lewis *et al.*, 2000; Forde *et al.*, 2008). Granulosa cell *BGCAN* expression levels were also similar between DF and SF, and this may show the lack of distinct follicle hierarchy in androgenised ewes, and possibly that the control SF group is more heterogeneous than the SF of a monitored follicle wave. However, *FST* mRNA expression was shown to be elevated in DF versus SF granulosa cells, contrary to follicular fluid concentrations within bovine DF (Austin *et al.*, 2001), while GLS analysis showed that INHBA mRNA levels were elevated in granulosa cells from SF. This may point to a potential emphasis on activin expression and function in follicles classified as SF, while counteracting activin in DF may be essential for further differentiation (Austin *et al.*, 2001).

No previous studies have investigated granulosa cell *CCND2* expression, as a proliferation marker gene, in abnormally large DF and SF recovered from prenatally androgenised ewes. Our results show that prenatal androgenisation by TP had no effect on the granulosa cell expression profile for *CCND2,* thus, the increased growth seen in the DF and SF recovered (**Chapter 3**) is not due to enhanced *CCND2* expression. However, it is possible that in largest follicles from androgenised ewes the stimulus for cell cycle progression (FSH and oestradiol) is limited by luteinisation factors (progesterone) signalling the exit of the cell cycle, as would normally occur in preovulatory follicles undergoing luteinisation (Richards *et al.*, 1998). This would overall lead to unaltered *CCND2* transcription. Proliferation is increased in granulosa cells recovered from unstimulated (no exogenous FSH) 4-8 mm follicles of anovulatory PCOS women (Das *et al.*, 2008), although granulosa cells were isolated from follicles at an earlier stage of development compared to the follicles used in this study. The study cited above used a different proliferation marker, namely Ki-67

(antigen KI-67), and it is possible that excess proliferation is driven by increased granulosa cell expression of *Ki-67* in androgenised females rather than the Cyclins. Therefore, *Ki-67* granulosa cell expression in DF and SF should be investigated in the future to determine if granulosa cell proliferation is increased through actions of Ki-67 (Endl & Gerdes, 2000). Expression levels of *CCND2* in granulosa cells were slightly increased in SF versus DF based on GLS analysis. This finding again highlights the fact that SF in this study may be a more heterogeneous group of follicles than SF monitored during DF selection, as granulosa cell *CCND2* expression was upregulated within bovine DF compared to SF (Mihm *et al.*, 2008). This finding is not due to prenatal TP treatment altering differential granulosa cell expression in DF and SF, as no prenatal treatment and follicle classification interaction was observed.

Prenatal androgenisation by TP had no effect on *MIF* granulosa cell expression in DF and SF. As MIF is a differentiation (for dominance and luteinisation) marker (Rath-Coursey *et al.*, 1999; Bove *et al.*, 2000), it was thought that prenatal exposure to excess TP *in utero* would increase *MIF* expression in DF and SF, which are larger and more steroidogenic than in controls. Despite this, expression levels of *MIF* were similar in granulosa cells from DF compared to SF, with no interaction between prenatal treatment and follicle classification detected. Therefore, differential MIF transcription does not appear to contribute to the abnormal differentiation of largest follicles recovered from androgenised ewes.

Prenatal androgenisation by TP had no effect on the transcription levels of a selection of pro- and anti-apoptotic genes within the granulosa cells of DF and SF. Expression levels of *BAX*, *CASP3* and *MCL-1* were similar between treatment groups. As androgenisation by TP results in excessive follicular growth (West *et al.*, 2001; Steckler *et al.*, 2007a), we would expect the apoptotic mechanisms in granulosa cells to be altered by prenatal androgenisation. Our results are in contrast to those found in anovulatory PCOS women. Studies in 4-8 mm follicles from PCOS patients investigating CASP3 localisation and expression in granulosa cells using immunohistochemistry and real-time PCR, determined that *CASP3* expression levels decreased in comparison to controls (Das *et al.*, 2008). The reduction in CASP3 levels does support the idea that granulosa cell apoptosis, at least in earlier stages of follicle development, is perhaps lowered and thus potentially prenatally programmed by androgen excess. Again, the Das study cited investigated an earlier stage of follicle development, thus perhaps another executioner of apoptosis is differentially expressed at later stages of follicle development in the prenatally androgenised ewe model.

In fact, the lack of differential mRNA expression of these three apoptotic marker genes could be as a result of the selection of inappropriate apoptotic markers investigated given there are many effectors of apoptosis (Amsterdam *et al.*, 2003; Choi *et al.*, 2004; Tsai *et al.*, 2005; D'Haeseleer *et al.*, 2006). However, the markers investigated in this study are considered to be the main players in granulosa cell apoptosis (Amsterdam *et al.*, 2003). For all three apoptotic markers investigated (*MCL-1*, *CASP3* and *BAX*), expression levels were similar between DF and SF. In carefully monitored bovine DF *MCL-1* expression in granulosa cells is upregulated compared to SF (Evans *et al.*, 2004). This again may indicate that DF and SF in our study were a lot closer in terms of apoptosis potential than DF and SF recovered following monitoring of a follicle wave. The lack of differential expression between DF and SF in the apoptotic genes investigated was independent of treatment, as there was no significant prenatal treatment with follicle classification interaction.

This chapter focused on the differential expression of a number of genes of interest. However, as not all transcripts are translated into functional protein, in order to conclusively prove that those transcripts differentially expressed actually alter the amount of protein present within granulosa cells a future study determining the protein levels of those genes (LHR, FSHR ex 4/5, FSHR exon 9/10 and HSD3B1) should be undertaken. This would be achieved by either western blotting from granulosa cell preparations isolated from DF and SF or by means of immunohistochemistry of whole DF and SF followed by semi-quantification of the protein levels with the aid of confocal microscopy and the use of the appropriate software similar to that described previously (Ortega *et al.*, 2009).

In conclusion, the results of this experiment have for the first time provided molecular evidence that prenatal androgenisation is able to program the granulosa cell transcription of large antral follicles possibly underlying the enhanced follicle differentiation and increased growth and steroidogenesis. Clearly, further studies are required to elucidate whether expression of other key genes is affected and causatively involved in the abnormal follicle development, and whether increased growth and steroidogenesis in DF and SF of prenatally androgenised ewes results from a differential response to the gonadotrophins or are due to intrinsic differences within granulosa cells.

CHAPTER 6. DOES PRENATAL PROGRAMMING BY EXCESS ANDROGENS ALTER THE INTRINSIC AND/OR GONADOTORPHIN-STIMULATED PRODUCTION OF OESTRADIOL AND PROGESTERONE BY GRANULOSA CELLS?

6.1 INTRODUCTION

Prenatal androgenisation by TP in sheep results in enhanced growth and steroidogenesis of DF and SF as detailed in **Chapter 3**. Furthermore, the molecular abnormalities revealed in **Chapter 5** of increased granulosa cell *LHR* mRNA expression together with decreased *FSHR* mRNA expression demonstrate enhanced DF and SF differentiation as a result of prenatal androgenisation through TP. Additionally, increased follicular progesterone production is likely related to elevated expression of *HSD3B1* mRNA in granulosa cells. However, it is still unknown how prenatal programming by TP results in increased oestradiol and progesterone synthesis within DF and SF and possibilities for increased granulosa cell steroidogenesis include (1) prenatal programming by TP altering the external hormonal environment such that peripheral concentrations of LH are increased (Sarma *et al.*, 2005) which then in turn drive the increased synthesis of both steroid hormones within granulosa cells (2) prenatal programming by TP alters granulosa cells themselves, such that they inherently produce increased amounts of steroid hormone, i.e. there is an inherent difference within large antral follicle granulosa cells regardless of the peripheral hormonal environment, or (3) a combination of (1) and (2). There is some evidence for this: PCOS, which may be due to prenatal androgenisation, results in an increase in inherent follicular somatic steroid production, as steroidogenic enzymes have been shown to be intrinsically upregulated in theca cells recovered from 3-5mm follicles from PCOS patients (Wickenheisser *et al.*, 2000).

The relationship between endocrine changes (such as LH and FSH secretion) and ovarian changes (such as granulosa cell steroid production) *in vivo* is complex, as one depends and responds to changes in the other (Duggavathi *et al.*, 2005). Cell culture provides an approach where the actions of specific factors can be determined independent of feedbacks, which means specifically for this study, that the putative differential effects of LH and FSH on granulosa cell steroid production from control and prenatally androgenised follicles can be explored. Previous efforts to develop a physiological granulosa cell culture system in animal models (ovine, bovine and rat) have been characterised by a consistent decline in oestradiol production with time in culture while progesterone production increases (Luck *et al.*, 1990; Meidan *et al.*, 1992; Spicer *et al.*, 1993; Gong *et al.*, 1994). These two characteristics are suggestive of the onset of luteinisation and do not mimick changes occurring during continued follicle growth and development. Previous cultures

have also used foetal calf serum in their culture system to facilitate the adhesion (and thus survival) of cells to the plate surface (Luck *et al.*, 1990; Langhout *et al.*, 1991; Spicer *et al.*, 1993; Gong *et al.*, 1994). However, foetal calf serum may introduce unknown contaminants, such as inhibin, growth hormone or other growth factors into the culture, which may affect cellular responsiveness to subsequent stimuli. Indeed, many previous culture systems employ relatively short-term cultures in which cells undergoing early luteinisation retain the ability to synthesize oestradiol (Gong *et al.*, 1994). Results from such studies can be misleading because the hormonal 'responses' to treatment may just reflect a change in the rate of luteinization. Therefore, we used a 6 day culture system developed for bovine (Gutierrez *et al.*, 1997) and ovine (Campbell *et al.*, 1996) granulosa cells which does not use foetal calf serum, and where the cellular phenotype of granulosa cells particularly from medium antral follicles is maintained. Within this culture system FSH can induce oestradiol production by bovine granulosa cells and this induction is related to an increase in *CYP19A1* mRNA expression (Manuel Silva & Price, 2000).

In order to determine if granulosa cells are intrinsically different between androgenised and control ewes (as suggested by the molecular changes), or whether the external (hormonal) environment causes the differences detected in antral follicle function, granulosa cells from DF and SF were cultured *in vitro* for 6 days under different gonadotrophin conditions, zero supplemented media, ovine FSH only, and human recombinant LH together with FSH. Oestradiol and progesterone production were investigated to address the following questions: Are granulosa cells from DF and SF from TP ewes inherently more steroidogenic independent of gonadotrophin stimulation, or more LH-, and less FSH-responsive in terms of steroid hormone synthesis; or do they show similar steroid hormone synthesis to cells from control ewes when exposed to the same hormone conditions. The aim was also to determine the expression profiles of *FSHR* and *LHR* together with the FSH and LH responsive genes *CYP19A1*, *HSD3B1* and *CCND2* following culture to determine if prenatal programming by androgenisation resulted in differential expression of these genes under gonadotrophin stimulation.

Additionally, as in **Chapter 3**, this study aimed to delineate the effects of prenatal androgenisation which arise as a result of oestrogenic action and those that are due to the action of androgens. This was achieved by replacing TP with DHT to induce prenatal androgenisation.

It is with these aims in mind that granulosa cells recovered from DF and SF of control and prenatally androgenised ewes (both by TP and DHT) were cultured in a serum-free culture system for 6 days in differing hormonal conditions. This study is referred to as the experimental culture throughout the chapter. To the best of our knowledge no group has investigated the effect of prenatal androgenisation on granulosa cell steroid production and the response to FSH and LH *in vitro*, removed from the effect of peripheral LH and FSH concentrations. Subsequently, and to address two issues arising from experimental cultures which related to differences in live cell numbers plated and the dose of FSH and LH needed for stimulation, granulosa cells obtained from follicles excised from abattoir sheep ovaries were cultured to determine (1) the effect of seeding different densities of granulosa cells on live cell numbers and viability over the 6 day culture period and (2) to determine what ovine LH (oLH) dose the recombinant human LH used in the experimental cultures relate to.

6.2 Materials and Methods

6.2.1 Experimental Culture of Granulosa Cells

Granulosa cells were obtained and cultured under serum-free conditions by a previously described method (Gutierrez *et al.*, 1997; Glister *et al.*, 2005). Ovaries from experimental year group 5 sheep (10 months of age) were recovered, the largest (generally two) antral follicles excised from the stroma and granulosa cells harvested from individual follicles as described previously in section 2.4. Granulosa cells were washed, seeded and cultured as detailed in section 2.8. Following the second wash, granulosa cells were re-suspended in 410µl of zero supplemented medium. Harvested cells were split into eight 50 µl aliquots and 6 were seeded into wells, thus different numbers of live cells were plated per well. The remaining 100 µl of harvested cells were stored for subsequent gene expression analysis and 10 µl used to determine live granulosa cell numbers. Trypan blue exclusion was used to determine live cell numbers before seeding and after 6 days of culture as described in section 2.9. Granulosa cells were cultured using three different culture media treatments: (1) 0 supplemented media (2) 1 ng oFSH (NHPP oFSH 20) and (3) 1 ng oFSH and 11.67 ng human recombinant LH (Serono Ltd, UK). Conditioned media was removed and replaced with fresh media every 48 hours as described in section 2.8. Ovaries collected from experimental group 5 sheep were collected over 3 days, therefore three independent cultures were used in the study. Duplicate wells were pooled for each timepoint (48 hrs, 96 hrs and 144 hrs) and for each media condition for subsequent steroid assay as described in section 2.6.

6.2.2 Control Cell Culture Set-Ups

Granulosa cell isolation and control culture to determine the effect of plating different cell densities on live cell numbers and viability

Ovine ovaries were collected from a local abattoir on three different days and placed in transport media (M199 medium, supplemented with 1% (vol/vol) antibiotic-antimycotic solution and 3 mM l-glutamine (all reagents from Sigma Ltd, Poole, UK). Approximately 40 follicles ≥5 mm in diameter were excised, follicular fluid was aspirated, and granulosa cells recovered as described in section 2.4. Granulosa cell preparations were pooled into 3 preparations prior to washing. Granulosa cells were washed, live cell numbers determined, and cultured as described in section 2.8, with two alterations. Firstly, due to the low numbers of granulosa cells recovered from individual follicles in the experimental culture, for all washes zero supplemented media was replaced with 0.1 M PBS to minimise the loss of granulosa cells during washing. Only following the second 0.1 M PBS wash, granulosa cells were re-suspended in 1 ml of supplemented zero media. Secondly, following determination of live granulosa cell numbers by Trypan blue exclusion (section 2.9), granulosa cells were seeded in a volume of 50 µl at various densities (32K/well, 16K/well, 8K/well and 4K/well) in wells containing 200 µl of media with or without gonadotrophin treatments. For each cell density, three different media conditions in duplicate were used, which included (1) 0 supplemented media (2) 1 ng oFSH (NHPP oFSH 20) supplemented media and (3) 1 ng oFSH and 11.67 ng human recombinant LH (Serono Ltd, UK) supplemented media. Conditioned media was removed every 48hours and replaced with fresh media as described in section 2.8. Conditioned media was stored at -20°C for subsequent steroid assay as described in section 2.6. Three independent cultures were carried out, and within each independent culture three 96 well plates were used to determine live cell numbers and viability at (1) 48 hrs (2) 96 hrs and (3) 144 hrs. Duplicate wells were pooled for each timepoint (48, 96 and 144hrs) and for each treatment (0, 1 ng FSH and 1 ng FSH + 11.67 ng human recombinant LH). After 48, 96 and 144hrs granulosa cells were harvested, counted using a haemocytometer and cell viability determined by Trypan blue exclusion (section 2.9).

Dose response to oFSH and oLH

Ovine ovaries were collected from a local abattoir on three different days and placed in transport media (M199 medium, supplemented with 1% (vol/vol) antibiotic-antimycotic solution and 3 mM l-glutamine (all reagents from Sigma Ltd, Poole, UK). Granulosa cells were harvested and pooled from ~40 follicles >5 mm in diameter as described in section 2.4. Granulosa cell preparations were generally pooled into 3 preparations prior to

washing. Granulosa cells were washed and live cell numbers and viability determined using Trypan blue exclusion as described in sections 2.8 and 2.9, respectively. Following determination of granulosa cell viability and live cell numbers, granulosa cells were seeded at 75,000 live cells/50 µl per well in 200 µl pre-equilibrated media. There were in total ten different treatments containing 1ng/ml oFSH (NHPP oFSH 20), and increasing concentrations of oLH (NIDDK oLH S26) and human recombinant LH (Serono Ltd, UK). The ten different treatments investigated were (1) 0 supplemented media, (2) 0.1 ng oFSH, (3) 1 ng oFSH, (4) 10 ng oFSH, (5) 100 ng oFSH, (6) 0.1 ng oLH + 1 ng oFSH, (7) 1 ng oLH + 1 ng oFSH, (8) 10 ng oLH + 1 ng oFSH, (9) 100 ng oLH + 1 ng oFSH and (10) 11.67 ng human recombinant LH + 1 ng oFSH. Conditioned media was removed at 48 hours and replaced with fresh media as described in section 2.8. Conditioned media was stored at -20°C for subsequent steroid assay as described in section 2.6. Three independent set-ups were carried out on three different days, and live numbers of granulosa cells and viability determined after 144 hours by Trypan blue exclusion (section 2.9).

6.2.3 Steroid Concentrations in Conditioned Media - RIA

Concentrations of the steroid hormones oestradiol and progesterone in a volume of 10-200 µl conditioned media were determined by validated radioimmunoassays using the assay procedure and reagents as detailed in section 2.6. Unextracted conditioned media from both experimental and control set-ups were included within the same assays. For the oestradiol assay, mean intra-assay and interassay coefficient of variation for one quality control sample at a concentration of 1.21 ± 0.13 pg/ml was 8.5 and 9% respectively. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 0.50 ± 0.04 pg/ml. For the progesterone assay, mean intra-assay and interassay coefficient of variation for one quality control sample at a concentration of 2.61 ± 0.33 ng/ml was 9 and 10.2% respectively. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 0.1 ± 0.02 ng/ml. The limit of assay sensitivity was assigned to those samples in which the concentration of hormone was below the sensitivity of the assay despite using the maximum volume available.

6.2.4 Molecular Biology

Total RNA isolated from granulosa cells before and after culture was isolated, DNase I treated and reverse transcribed using the methods detailed in section 2.7. Due to the recovery of a very small amount of total RNA from some individual follicles, as a result of a lack of granulosa cells following pelleting, only a subgroup of granulosa cell preparations

were selected to act as negative controls within this year group. The quantity and quality of cDNA and RNA were assessed using spectrophotometry, as detailed in section 2.7. The quantitative real-time PCR method used to measure gene expression within granulosa cells recovered from DF and SF before and after culture and the primer pairs used are detailed in section 2.7 and Table 5.1, respectively. Total RNA yield from cultured granulosa cells varied between 1-600 ng/µl, 500 ng of tRNA was reverse transcribed, while 400 ng of cDNA was added to the PCR reaction mixture. In spite of this seemingly high concentration of cDNA added to the reaction mix, not many cultured samples obtained a sufficiently low cycle threshold number for *ACTB* and the genes of interest (*CYP19A1*, *LHR* and *CCND2*). The cDNA concentration and quality of a selected few samples after 6 days of culture following RNA extraction and reverse transcription, together with the cycle threshold achieved for *ACTB* are shown in Table 6.1.

Follicle ID	Prenatal Treatment	cDNA Concentration (ng/µl)	A260:280	A260:230	*ACTB* cycle threshold number
21SF – 0	TP	732	1.82	1.53	31.46
21SF – FSH	TP	734	1.83	1.19	32.72
21SF – LH&FSH	TP	1245	1.8	1.64	34.33
27DF – 0	TP	470	1.52	1.2	32.19
27DF – FSH	TP	463	1.69	0.99	32.08
27DF –LH&FSH	TP	508	1.8	1.69	30.92
33DF – 0	DHT	1061	1.76	1.34	36.03
33DF – FSH	DHT	1328	1.81	2.22	35.12
33DF – LH&FSH	DHT	1019	1.82	1.51	35.87
4DF – 0	Control	930	1.8	1.25	33.78
4DF – FSH	Control	1050	1.8	1.02	33.98
4DF – LH&FSH	Control	1087	1.81	1.33	34.67

Table 6.1 The quality and quantity of cDNA from selected cultured granulosa cells after 6 days of culture isolated from dominant (DF) or subordinate(DF) follicles following reverse transcription of 500 ng of total RNA. Also presented is the *ACTB* the cycle threshold number achieved after real-time PCR amplification. A260 = absorbance at 260 nm, A230 = absorbance at 230 nm, *ACTB* = Beta Actin, TP = testosterone propionate, DHT = 5α-dihydrotestosterone.

6.2.5 Statistical Analyses

The results for follicle size, intrafollicular concentrations of oestradiol and progesterone, the oestradiol to progesterone ratio (Chapter 3) and expression of mRNA encoding *LHR*, *FSHR* exon 9/10, *CYP19A1* and *HSD3B1* in granulosa cells of DF and SF recovered from each ewe from Year Group 5 have been described previously (sections 3.3.4 and 5.3). The results presented in this chapter for these dependent variables are solely on the effect of

prenatal treatment and follicle classification. This allows the results obtained *in vitro* to be compared to those seen *in vivo*.

For individual follicle parameters (follicle size, follicular fluid oestradiol concentration, follicular fluid progesterone concentration and follicular fluid oestradiol: progesterone ratio) a two-way AMOVA with prenatal treatment (Control, TP, and DHT) and follicle classification (DF or SF) as the two factors was used to determine any influences on follicle function. In addition, any logical interactions between factors were included in the model. A Tukey's posthoc test was used to determine significant differences between levels within a factor.

A two-way ANOVA, with prenatal treatment and follicle classification as the two factors was used to determine any influences on granulosa cell mRNA expression for *LHR*, *FSHR* exon 9/10, *CYP19A1* and *HSD3B1* in year group 5 (10 month old) sheep only. In addition, any interaction between the two factors was determined. A Tukey's posthoc test was used to determine significant differences between levels within a factor.

A two-way ANOVA with follicle classification (DF or SF) and prenatal treatment (control, TP and DHT) as the two factors was used to determine any difference in the number of granulosa cells plated per individual follicle. Any interaction between follicle classification and prenatal treatment was also investigated. A Tukey's posthoc test was used to determine significant differences between levels within a factor

In vitro steroid production of oestradiol and progesterone from experimental granulosa cells at 48, 96 and 144 hours was initially normalised to production per 1000 live cells plated. Data were assessed using a GLM with day of culture setup (three independent days, 1-3), prenatal treatment (Control, TP and DHT), media conditions (0, 1 ng oFSH and 11.67 ng human Rec LH + 1 ng oFSH), culture time-point (48, 96 and 144hrs) and follicle classification (DF and SF, see explanations above) as the fixed factors potentially influencing the results. Interactions were also considered in this analysis. If there were more than 2 levels to a factor, a Tukey's posthoc test was used to determine significant differences between levels within that factor. Because the factor 'follicle' is known to contribute to the variation in results, but could not be included in the GLM analyses, a generalised least square (GLS) model was also applied to the data with the same fixed factors as for the GLM analyses, but with 'follicle' included as a random factor to determine whether this altered the outcome of GLM analyses. Again results from the GLM

and GLS analyses are consistent, with few exceptions, for example, where only the GLS analysis highlighted a significant effect of the combined gonadotrophin treatment on *in vitro* progesterone production. In addition, steroid production between 96-144 hours was normalised to 1,000 live cells counted after 6 days of culture, which was only possible in a very reduced number of samples, and analysed using the same GLM model but without culture time-point as a fixed factor.

To determine relative changes in steroid production over time or with gonadotrophin supplementation, the percent change in oestradiol or progesterone production normalised to 1,000 live cells plated was calculated 1) from 48 to 96 hrs, and from 96 to 144 hrs separately for each media condition, and 2) following FSH or FSH+LH supplementation of the basic medium for each time-point (48 hrs, 96 hrs and 144hrs). A student t-test was used to determine any differences in relative changes in steroid production between cells from control and androgenised (TP and DHT) ewes.

To assess granulosa cell viability data was expressed as (1) absolute live cell numbers and (2) a percentage - the number of live cells divided by the total number of cells (live and dead) at each time point. A general linear model (GLM) was utilised, percentage cell viability being the dependent variable and with cell density (32K, 16K, 8K and 4K), media conditions (0, 1 ng oFSH only and 1 ng oFSH + 11.67 ng human recombinant LH) and culture time-point (48 hrs, 96 hrs and 144 hrs) as the three fixed factors, and any interactions between these factors was determined. Any differences between levels within a factor were determined by Tukey post-hoc test.

To assess the effect of oFSH and oLH on granulosa cell steroid production (both oestradiol and progesterone), data were expressed as pg/10000 live cells plated (oestradiol) and ng/10000 live cells plated (progesterone). A general linear model was utilised with culture time-point (48 hrs, 96 hrs and 144 hrs) and media treatments for FSH dose (0, 0.1 ng oFSH, 1 ng oFSH, 10 ng oFSH and 100 ng oFSH) or LH dose response (1 ng oFSH plus 0.1 ng oLH, 1 ng oLH, 10 ng oLH, 100 ng oLH and 11.67 ng human recombinant LH) as the two fixed factors, together with any interactions between these factors. Any differences between levels within factors were determined by Tukey post-hoc test.

6.3 RESULTS

6.3.1 Dominant and Subordinate Follicle Characteristics – Year Group 5

6.3.1.1 Follicle Size

Prenatal androgenisation by TP increased follicle diameter of the two largest follicles compared to controls (Figure 6.1). Androgenisation by DHT led to intermediate follicle diameters, which did not differ from diameters measured in control or TP-treated ewes. There was no overall difference in follicle diameter between follicles classified as DF or SF.

6.3.1.2 Follicular Fluid Oestradiol Concentration

Prenatal androgenisation by TP increased follicular fluid oestradiol concentration overall in both DF and SF compared to control and DHT-treated animals (Figure 6.1). Androgenisation by DHT had no effect on follicular fluid oestradiol concentration compared to controls. Follicular fluid oestradiol concentrations were also increased in DF versus SF, which confirms the follicle classification and suggests a higher health status in the DF (Evans & Martin, 2000).

6.3.1.3 Follicular Fluid Progesterone Concentration

Prenatal androgenisation by TP increased follicular fluid progesterone concentration overall (DF and SF) compared to control and DHT-treated animals (Figure 6.1). Androgenisation by DHT had no effect on follicular fluid progesterone concentration compared to controls. Follicular fluid progesterone concentrations were similar between DF and SF.

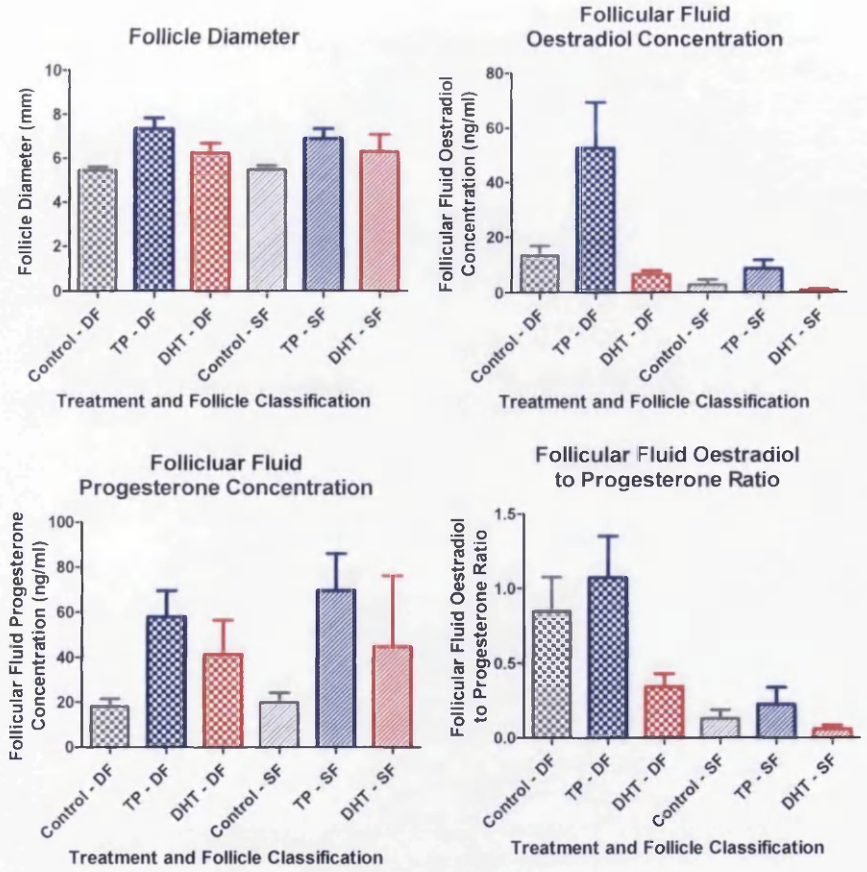
6.3.1.4 Follicular Fluid Oestrogen to Progesterone Ratio

Prenatal androgenisation had no effect on the follicular fluid oestradiol to progesterone ratio (Figure 6.1). The oestradiol to progesterone ratio was increased in follicles classified as DF versus SF, confirming our functional classification (Evans & Martin, 2000).

6.3.1.5 Expression of mRNA encoding *LHR*, *FSHR* exon 9/10, *CYP19A1* and *HSD3B1* in granulosa cells of DF and SF recovered from each ewe from Year Group 5 only.

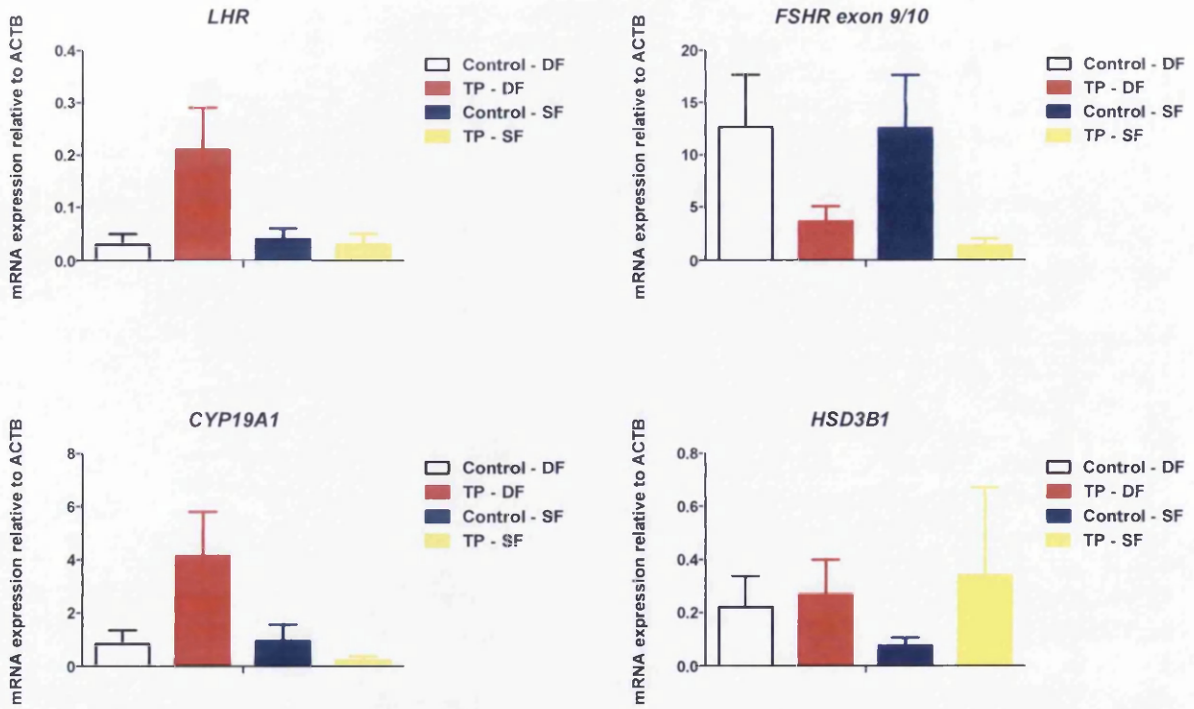
Prenatal androgenisation by TP reduced granulosa cell *FSHR* exon 9/10 mRNA expression within DF and SF (Figure 6.2). Prenatal androgenisation by TP had no effect on *LHR*, *CYP19A1* and *HSD3B1* granulosa cell mRNA expression. However, there was a tendency ($P = 0.1$) for a treatment with follicle classification interaction for *LHR* and *CYP19A1* transcript levels; specifically, granulosa cell transcript levels were similar between DF and

SF of controls, while DF transcript levels were higher in follicles from androgenised ewes. Granulosa cells mRNA expression levels of *LHR*, *FSHR* exon 9/10 and *HSD3B1* were similar between DF and SF, while *CYP19A1* expression was higher in DF compared to SF, supporting the follicular fluid results and our follicle classification (Figure 6.2). The analysis table listing the P values determined for each factor is shown in Figure 6.2.



Follicle Characteristic	Prenatal Treatment			GLM P value - Follicle Classification	GLM P valu - Prenatal Treatment* Follicle Classificatio
	GLM P value	Comparison within a factor	Tukey P value		
Follicle Diameter	<0.001	C vs TP	0.002	0.621	0.847
		C vs DHT	0.224		
		TP vs DHT	0.160		
Follicular Fluid Oestradiol Concentration	<0.001	C vs TP	0.002	0.000	0.769
		C vs DHT	0.647		
		TP vs DHT	<0.001		
Follicular Fluid Progesterone Concentration	<0.001	C vs TP	<0.001	0.874	0.952
		C vs DHT	0.39		
		TP vs DHT	0.050		
Follicular Fluid Oestradiol to Progesterone Ratio	0.604	C vs TP	0.993	0.000	0.291
		C vs DHT	0.653		
		TP vs DHT	0.601		

Figure 6.1 Follicle characteristics (follicle diameter, follicular fluid oestradiol concentration, follicular fluid progesterone concentration and follicular fluid oestradiol: progesterone ratio) of dominant (DF) and subordinate follicles (SF) where granulosa cells were subsequently cultured from 10 month old lambs (year group 5 only) prenatally exposed to testosterone propionate (TP) or 5α-dihydrotestosterone (DHT) or untreated (control-C). Values presented are means ± SEM. GLM P Values for each factor and levels within a factor are presented in the table. DF – dominant follicles; SF – subordinate follicles. DF – C = 11, TP = 14, DHT = 6; SF – C = 9, TP = 12, DHT = 3.



Gene	Prenatal Treatment	Follicle Classification	Prenatal Treatment/ Follicle Classification Interaction
LHR	0.346	0.501	0.107
FSHR exon 10	0.035	0.643	0.465
CYP19A1	0.825	0.049	0.106
HSD3B1	0.760	0.816	0.282

Figure 6.2 Mean *LHR*, *FSHR* exon 9/10, *CYP19A1* and *HSD3B1* mRNA expression in granulosa cells of dominant (DF) and subordinate (SF) follicles recovered from control (C) and prenatally androgenised (TP) ewes in 10 month old lambs (year group 5 only). Values presented are mean ± SEM. P Values for each factor are presented in the table (DF: C = 7; TP = 6 follicles – SF: C = 4; TP = 4 follicles). ACTB = β-Actin.

6.3.2 Number of Live Cells Plated Across Prenatal Treatment Groups

The number of live granulosa cells plated per treatment group (control, TP and DHT) per follicle classification (DF and SF) is shown in Figure 6.3. There was no difference in the number of live granulosa cells plated due to treatment group (P = 0.135). The number of live granulosa cells plated per follicle classification is shown in Figure 6.4. There was no difference in the number of live granulosa cells plated in DF compared to SF (P = 0.519). No interaction between prenatal treatment and follicle classification was determined.

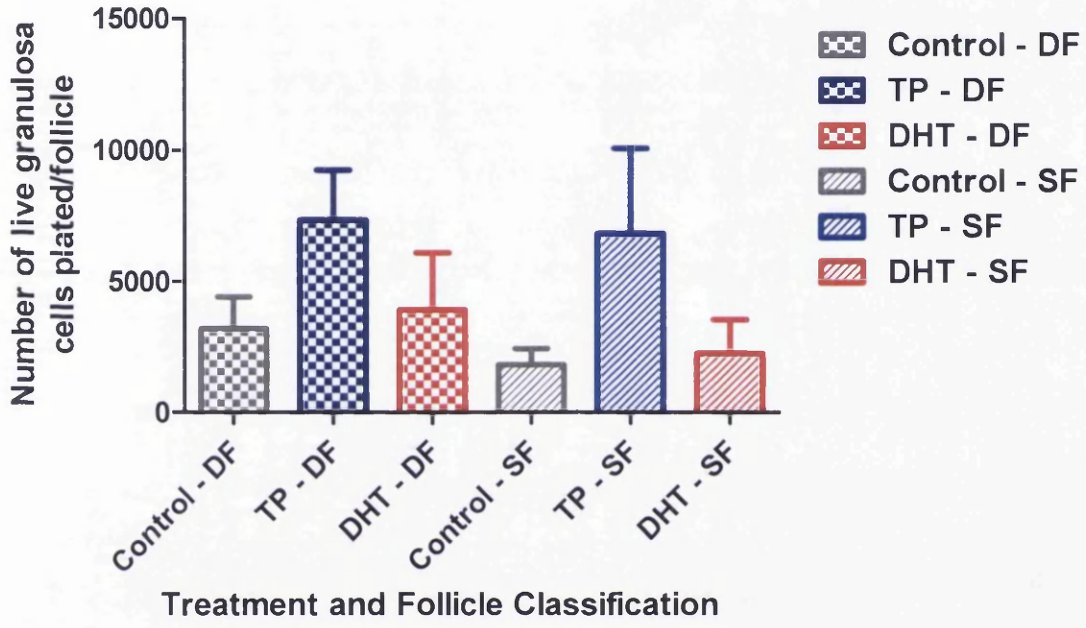


Figure 6.3 Average number of live granulosa cells plated per follicle per prenatal treatment group (control C, TP and DHT). Follicles were classified as either dominant (DF) or subordinate (SF) based on follicular fluid oestradiol concentration. (TP = testosterone propionate, DHT = 5α-dihydrotestosterone). Values presented are mean ± SEM. DF – C = 14, TP = 15, DHT = 8; SF – C = 12, TP = 13, DHT = 4.

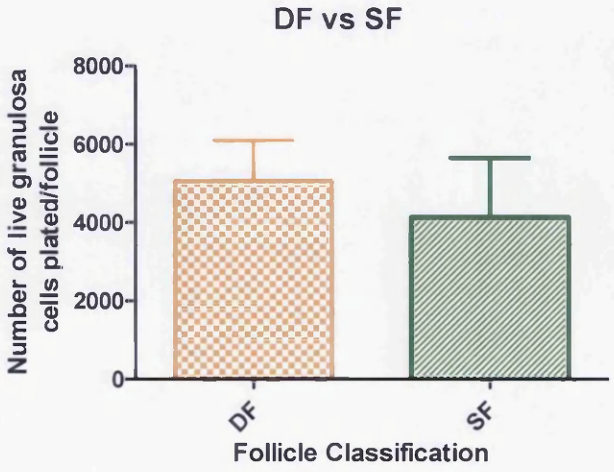


Figure 6.4 Average number of live granulosa cells plated per follicle when follicles were classified as either the dominant (DF) or subordinate follicle (SF) and data combined accross treatment groups Values presented are mean ± SEM. DF = 36 follicles, SF = 29 follicles.

6.3.3 Experimental Granulosa Cell Culture

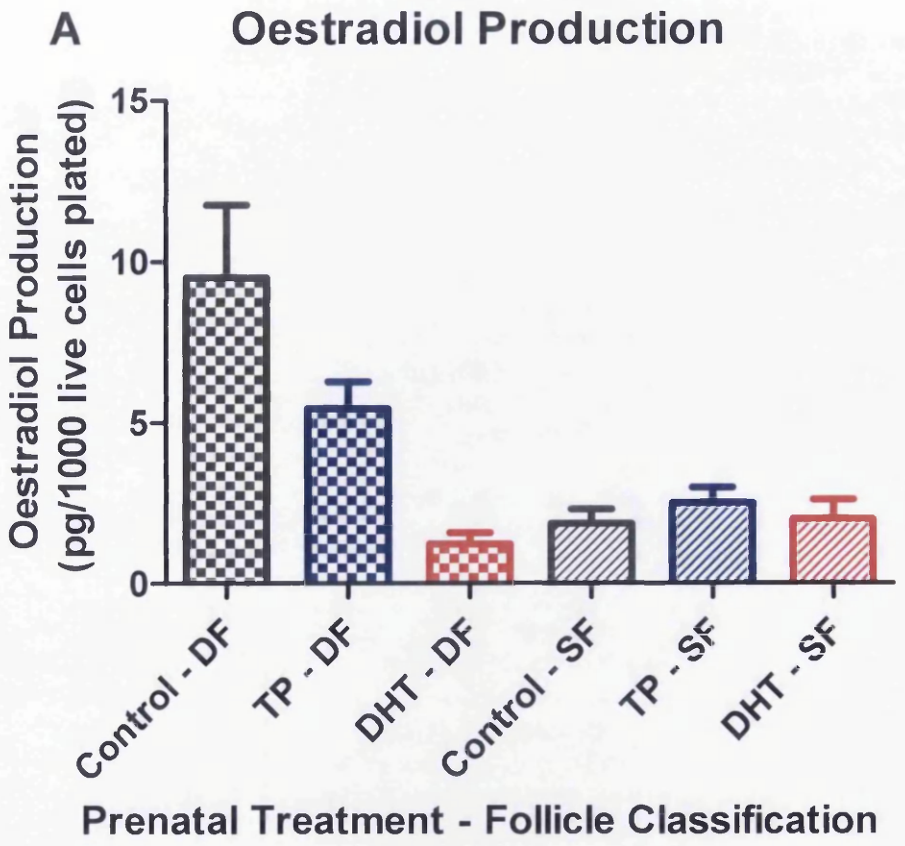
Granulosa Cell Oestradiol Production – Data normalised to 1,000 live granulosa cells plated

Prenatal treatment with DHT reduced oestradiol production by granulosa cells from the two largest follicles *in vitro*, in comparison to cells from follicles of both control ($P < 0.001$) and TP-treated ($P < 0.001$) ewes independent of gonadotrophin addition to media (Figure 6.5). However, there was no difference in the *in vitro* granulosa cell oestradiol production between follicles from control and TP-treated ewes. Granulosa cell oestradiol production normalised to the number of cells plated declined with time over the 6 days of culture ($P < 0.001$). Granulosa cell oestradiol production was higher in those follicles classified as DF compared to those classified as SF, confirming the functionality of the classification originally based on follicular fluid oestradiol. There was an interaction between prenatal treatment and follicle classification ($P = 0.008$), with, oestradiol production increased in granulosa cells from SF compared to DF from DHT-treated animals, while granulosa cell oestradiol production was reduced in SF compared to DF from both control and TP-treated ewes (Figure 6.6). Oestradiol production increased when FSH was added ($P = 0.017$), therefore, granulosa cells were overall FSH responsive during culture: however, the addition of LH did not enhance oestradiol production further. There was no media*prenatal treatment interaction, therefore, differences in gonadotrophin receptor mRNA expression in TP cells did not translate into a different oestradiol response to gonadotrophins *in vitro*. The day that the granulosa cell culture was set-up had a significant effect on oestradiol production (Table 6.2); oestradiol synthesis was higher from set-up 1 compared with set-ups 2 ($P = 0.015$) and 3 ($P = 0.004$). A summary of the results relating to oestradiol production is shown in Figure 6.5.

Day of Culture	Number of Control Follicles	Number of TP Treated follicles	Number of DHT Treated Follicles	Total
1	0	10	4	14
2	7	8	3	18
3	13	8	2	21

Table 6.2 The number of follicles recovered and cultured from each prenatal treatment group on the three separate days ovaries were removed and follicles recovered from experimental sheep. TP = testosterone propionate, DHT = 5α-dihydrotestosterone.

A



B

Comparisons of levels within factors	GLM P value
Day of culture 1 vs 2	0.015
Day of culture 1 vs 3	0.004
Day of culture 2 vs 3	0.476
Media – 0 vs FSH	0.017
Media – 0 vs LH&FSH	0.075
Media - FSH vs FSH&LH	0.832
Prenatal Treatment – Control vs TP	0.192
Prenatal Treatment – Control vs DHT	0.000
Prenatal Treatment – TP vs DHT	0.000
DF vs SF	0.000
48hrs vs 96hrs	0.001
48hrs vs 144hrs	0.001
96hrs vs 144hrs	0.002
Media*Prenatal Treatment	0.523
Prenatal Treatment*Follicle Classification	0.008

Figure 6.5 (**A**) Mean oestradiol production over a 6 day serum free culture system by granulosa cells of follicles classified as either dominant (DF) or subordinate (SF), recovered from prenatally androgenised (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) and control ewes. Production is combined across all culture time-points, day of culture set-ups and media conditions. Values presented are means ± SEM (**B**) P values obtained following GLM analysis for levels compared within a factor. Day of culture refers to the start date of three independent cultures (1 = day 1, 2 = day 2 and 3 = day 3), 0 media = no gonadotrophin supplementation, FSH = 1 ng FSH supplemented media, FSH&LH = 1 ng FSH & 11.67 ng recombinant human LH supplemented media. GLM = general linear model. DF – C = 11, TP = 14, DHT = 6; SF – C = 9, TP = 12, DHT = 3 follicles.

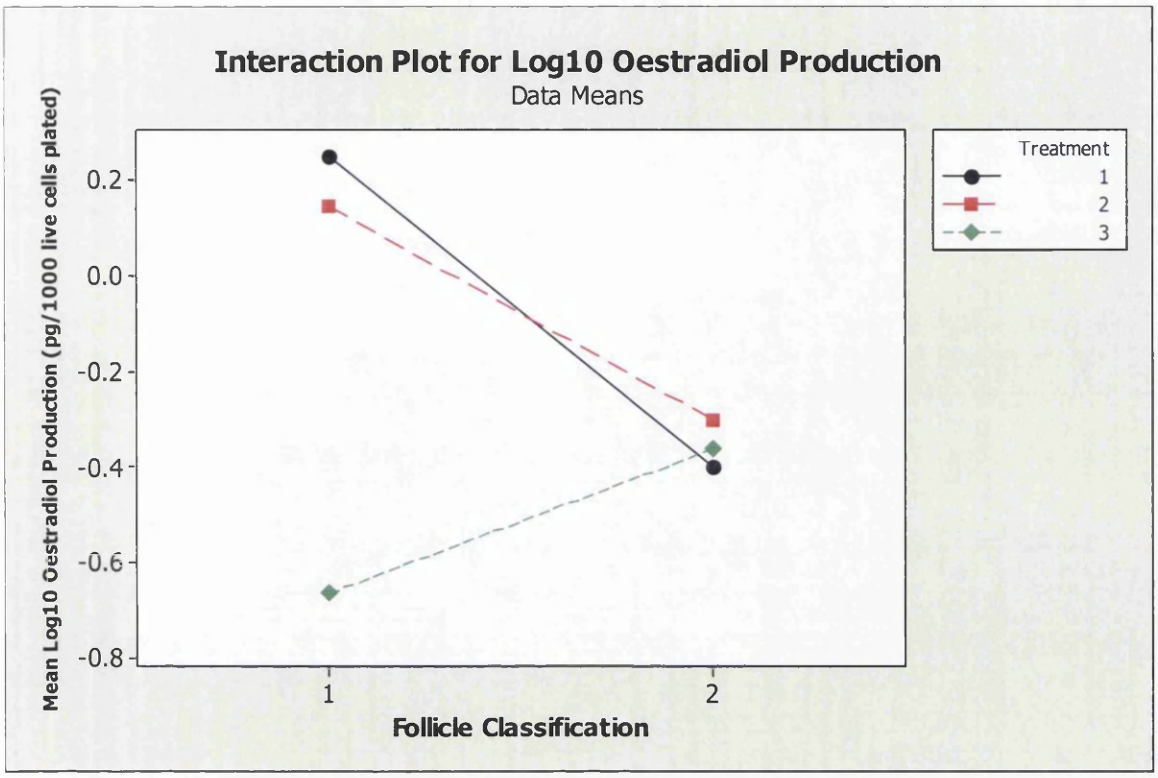
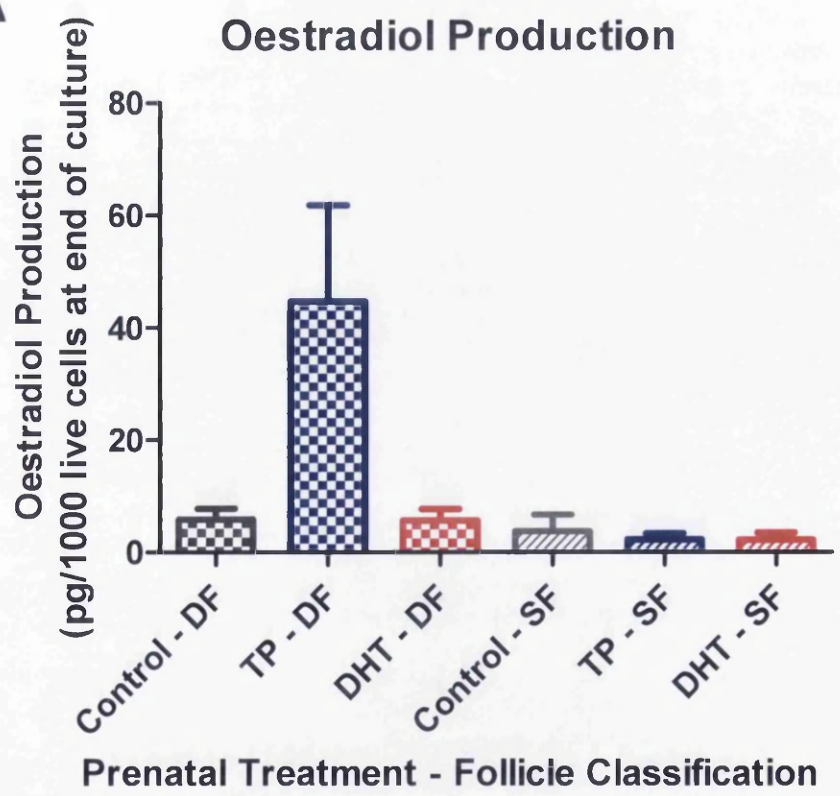


Figure 6.6 Interaction plot between follicle classification (dominant (DF) or subordinate (SF) follicle) and prenatal treatment (control, testosterone propionate (TP) and 5α-dihydrotestosterone (DHT)) for mean granulosa cell oestradiol production over a six day serum-free culture. Follicle classification - 1 = DF, 2 = SF. Treatment classification – 1 = control, 2 = TP and 3 = DHT.

Granulosa Cell Oestradiol Production – Data normalised to 1,000 live granulosa cells counted after 6 days of culture

Prenatal androgenisation by DHT had no effect on oestradiol production by live granulosa cells at 144hrs *in vitro* compared with control cells (Figure 6.7). However, there was a low tendency ($P = 0.1$) for TP cells to produce more oestradiol compared with control and DHT cells. Granulosa cell oestradiol production was higher in those follicles classified as DF compared to those classified as SF ($P < 0.001$). There was no interaction between prenatal treatment and follicle classification. Oestradiol production increased when FSH was added ($P = 0.034$), therefore, granulosa cells were overall FSH responsive during culture, however, the addition of LH to FSH reduced oestradiol production compared to FSH only media ($P = 0.024$). There was no media*prenatal treatment interaction, therefore, differences in gonadotrophin receptor mRNA expression in TP cells did not translate into a different oestradiol response to gonadotrophins *in vitro*. The day that the granulosa cell culture was set-up had a significant effect on oestradiol production, granulosa cell oestradiol synthesis was higher from set-up 1 compared with the other two days ($P = 0.003$ and 0.008, respectively).

A



B

Comparisons of levels within factors	GLM P value
Day of culture 1 vs 2	0.003
Day of culture 1 vs 3	0.008
Day of culture 2 vs 3	0.878
Media – 0 vs FSH	0.034
Media – 0 vs LH&FSH	0.561
Media - FSH vs FSH&LH	0.024
Prenatal Treatment – Control vs TP	0.123
Prenatal Treatment – Control vs DHT	0.672
Prenatal Treatment – TP vs DHT	0.102
DF vs SF	0.000
Media*Prenatal Treatment	0.573
Prenatal Treatment*Follicle Classification	0.457

Figure 6.7 (**A**) Mean oestradiol production between 96 and 144 hours of culture from follicles classified as either dominant (DF) or subordinate (SF), recovered from prenatally androgenised (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) and control ewes. Production is combined across all day of culture set-ups and media conditions. Values presented are means ± SEM (**B**) P values obtained for when levels were compared within a factor. Day of culture refers to the start date of three independent cultures (1 = day 1, 2 = day 2 and 3 = day 3), 0 media = no gonadotrophin supplement, FSH = 1 ng FSH supplemented media, FSH&LH = 1 ng FSH & 11.67 ng LH supplemented media. GLM = general linear model. DF – C = 5, TP = 7, DHT = 4; SF – C = 3, TP = 5, DHT = 3 follicles.

Percent change in Granulosa Cell Oestradiol Production with time in culture

Prenatal androgenisation by TP and DHT maintained granulosa cell oestradiol production (normalised to the number of cells plated) from 96 to 144hrs in unsupplemented medium compared to a severe drop when control cells were cultured under the same conditions ($P < 0.05$) (Figure 6.8). Similarly, prenatal androgenisation by TP maintained granulosa cell oestradiol production from 96 to 144hrs in culture media supplemented with FSH&LH compared to a severe drop when control cells were cultured in the same medium ($P < 0.05$) (Figure 6.8).

Percent change in Granulosa Cell Oestradiol Production with addition of gonadotrophins to the media

At 96 and 144hrs control cells increased their oestradiol production (normalised to the number of cells plated) 3 and 7-fold, respectively, in response to FSH, which was a higher percent change than that seen following culture of cells from androgenised ewes (Figure 6.9). The addition of LH to FSH had no effect on the percent change in granulosa cell oestradiol production relative to zero supplemented media at any of the culture time-points, nor were there differences between treatments in this response (data not shown).

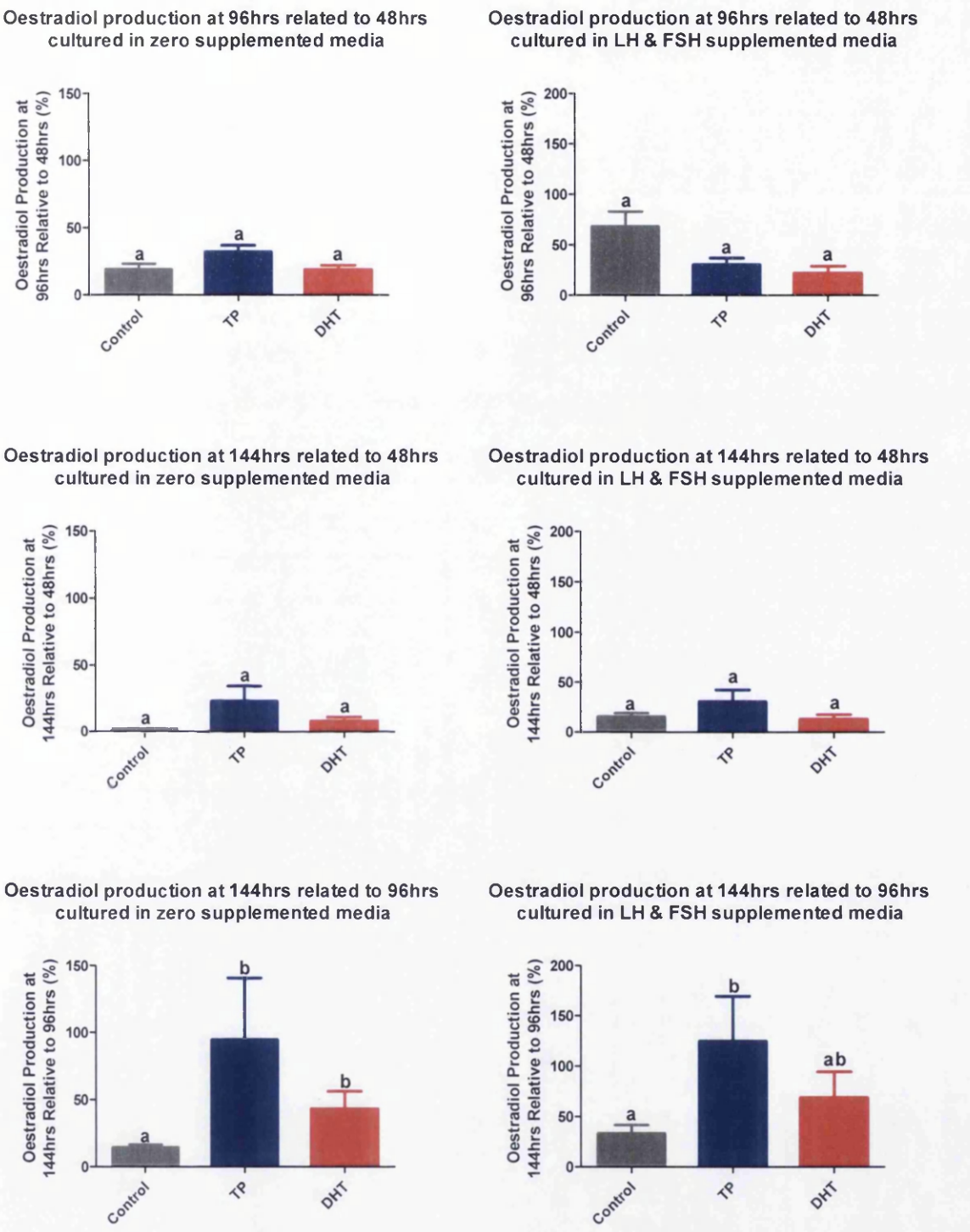


Figure 6.8 Effect of prenatal androgenisation by TP (testosterone propionate) and DHT (5α-dihydrotestosterone) on granulosa cell oestradiol production *in vitro*. Oestradiol production is related to the production at 48 and 96hrs, respectively, in the same media conditions. Oestradiol production is combined from both follicle classifications. Values presented are mean ± SEM. [a,b.] Between treatment groups, means without a common superscript differ ($P < 0.05$). Control = 20, TP = 26, DHT = 9 follicles.

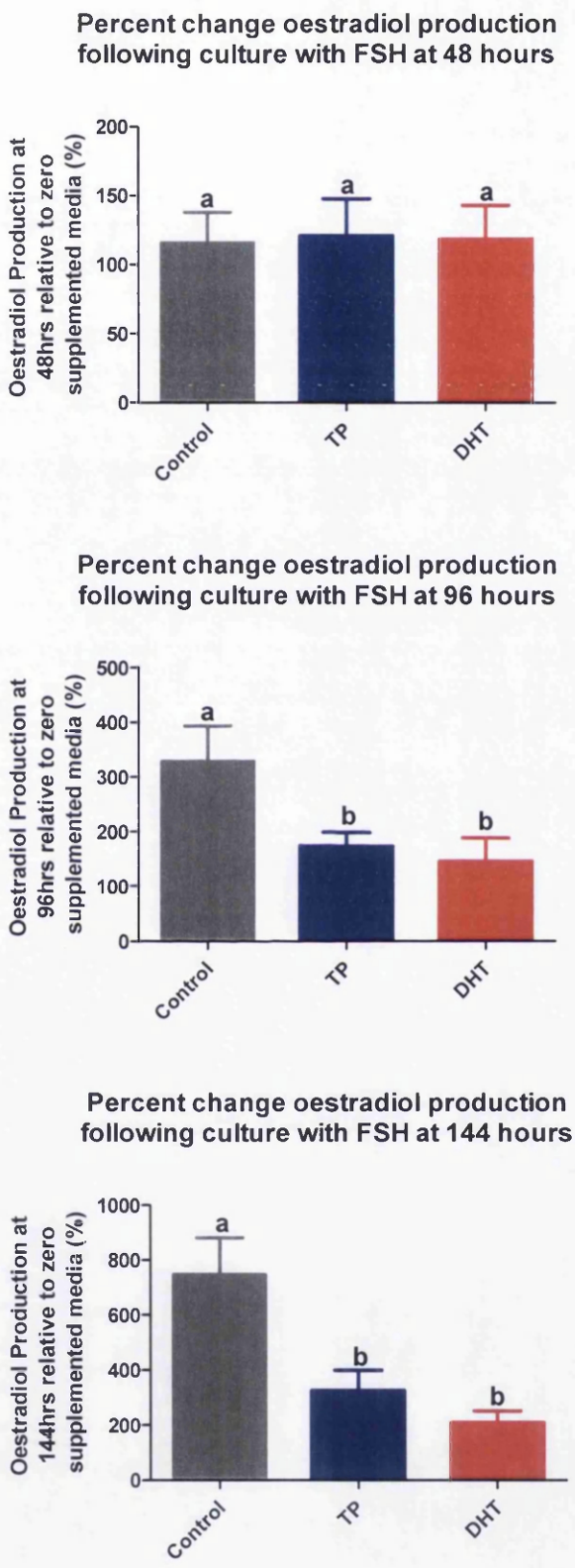
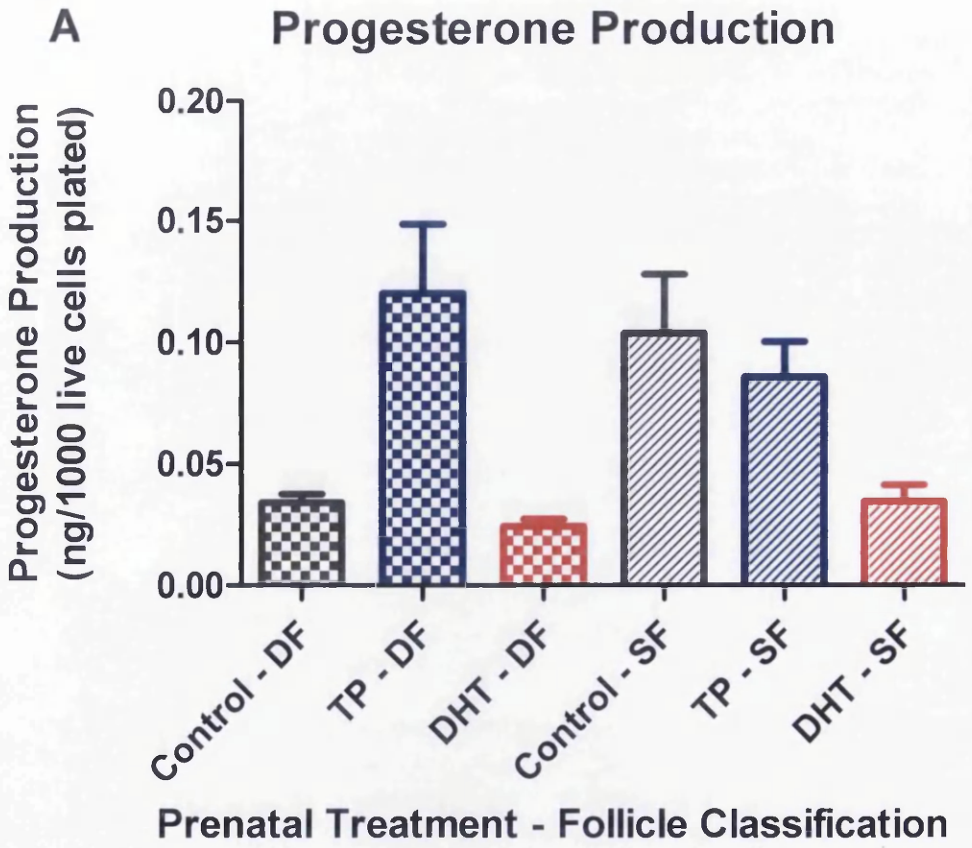


Figure 6.9 Effect of FSH on relative oestradiol production *in vitro,* by granulosa cells recovered from control ewes and ewes prenatally androgenised by TP (testosterone propionate) and DHT (5α-dihydrotestosterone). Oestradiol production is related to the number of cells plated and then expressed relative to zero supplemented media at each culture time-point (48, 96 and 144hrs). Oestradiol production is combined from both follicle classifications. Values presented are mean ± SEM. [a,b] Within a treatment group, means without a common superscript differ ($P < 0.05$). Control = 20, TP = 26, DHT = 9 follicles.

Granulosa Cell Progesterone Production – Data normalised to 1,000 live granulosa cells plated

Prenatal androgenisation by TP tended ($P = 0.1$) to enhance granulosa cell progesterone production compared to controls independent of gonadotrophin addition to the medium. Androgenisation with DHT tended ($P = 0.1$) to reduce granulosa cell progesterone production compared to controls, and significantly reduced progesterone production from the two largest follicles compared with TP prenatal treatment ($P = 0.009$). Analysis using GLS (but not GLM) showed that LH added to FSH enhanced progesterone production when compared with the zero culture medium ($P < 0.001$). Despite progesterone concentrations in media being normalised to cells plated at 0 hours and live cell numbers declining with time in culture (see Figure 6.10) no significant drop in progesterone concentrations in media was found with time in culture indicating an actual increase in progesterone production by surviving cells. In fact, GLS (but not GLM) analysis showed a significant increase in progesterone media concentrations ($P = 0.014$) between 48 and 144 hours. An interaction between prenatal treatment and follicle classification was determined, progesterone production from granulosa cells of DF and SF recovered from TP-treated ewes was more similar than progesterone production by cells from DF and SF recovered from the control and DHT groups ($P = 0.023$) (Figure 6.11). Granulosa cell progesterone production was increased in SF compared to DF ($P = 0.006$). There was no media*prenatal treatment interaction, therefore, differences in gonadotrophin receptor mRNA expression in TP cells did not translate into a different progesterone response to gonadotrophins *in vitro*. The day that the granulosa cell culture was set-up had a significant effect on progesterone synthesis, production was increased in the culture set up on day 2 compared to both 1 ($P = 0.008$) and 3 ($P < 0.001$).

A



B

Comparisons of levels within factors	GLM P value
Day of culture 1 vs 2	0.008
Day of culture 1 vs 3	0.534
Day of culture 2 vs 3	0.000
Media – 0 vs FSH	0.982
Media – 0 vs LH&FSH	0.23 4(GLS - 0.000)
Media - FSH vs FSH&LH	0.323
Prenatal Treatment – Control vs TP	0.131
Prenatal Treatment – Control vs DHT	0.151
Prenatal Treatment – TP vs DHT	0.009
DF vs SF	0.006
48hrs vs 96hrs	0.991
48hrs vs 144hrs	0.412 (GLS - 0.014)
96hrs vs 144hrs	0.332
Media*Prenatal Treatment	0.724
Prenatal Treatment*Follicle Classification	0.023

Figure 6.10 (A) Mean average granulosa cell progesterone production over a 6 day serum free culture system from follicles classified as either dominant (DF) or subordinate (SF), recovered from prenatally androgenised (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) and control ewes. Production is combined across all culture time-points, day of culture set-ups and media conditions. Values presented are means ± SEM (B) P values obtained following GLM analysis when levels were compared within a factor. Day of culture refers to the start date of three independent cultures, (1 = day 1, 2 = day 2 and 3 = day 3), 0 media = no gonadotrophin supplement, FSH = 1 ng FSH supplemented media, FSH&LH = 1 ng FSH & 11.67 ng LH supplemented media. GLM = general linear model, GLS = generalised least squares. DF – C = 11, TP = 14, DHT = 6; SF – C = 9, TP = 12, DHT = 3 follicles.

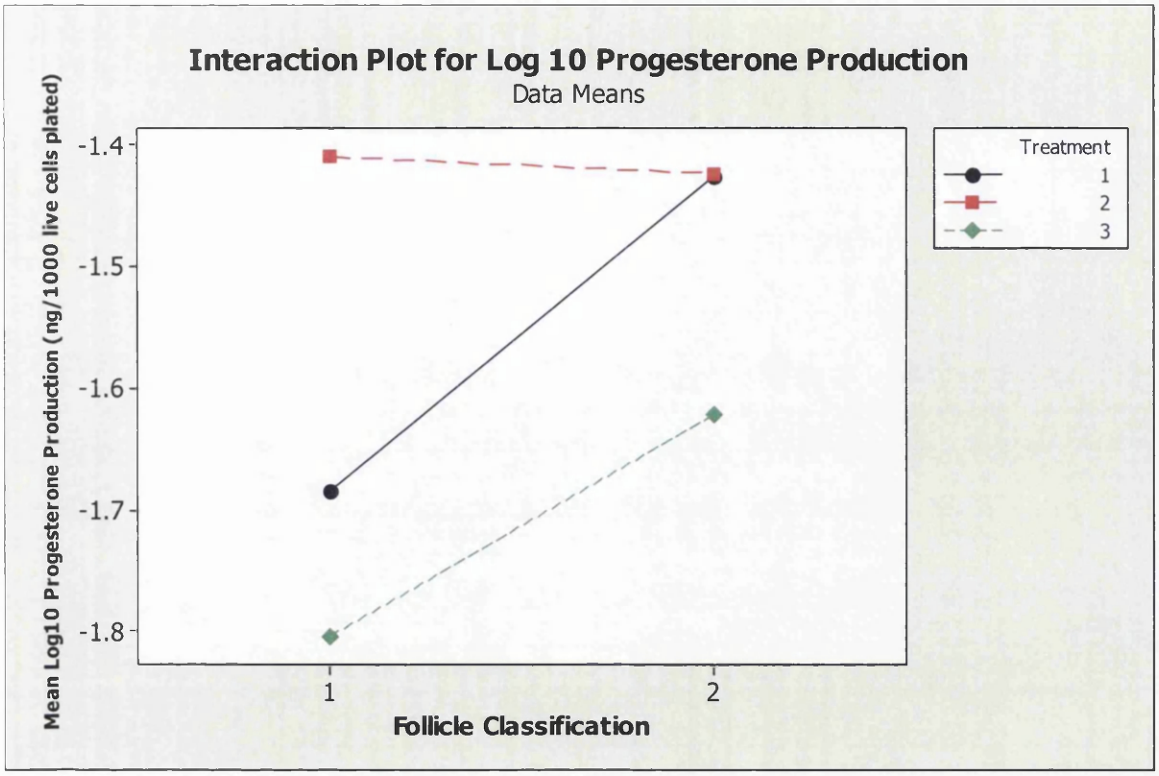
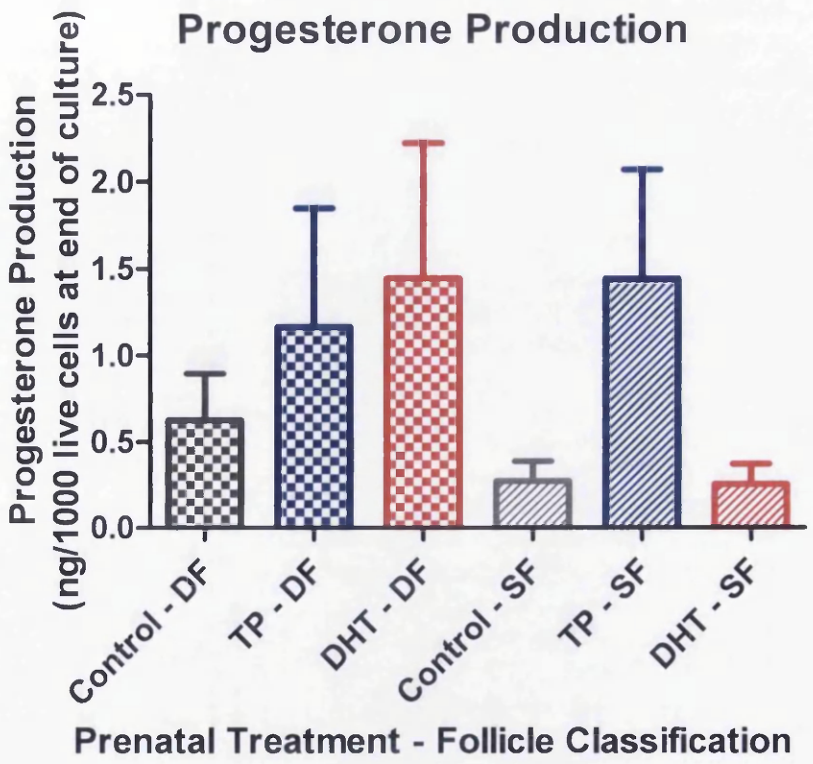


Figure 6.11 Interaction plot between follicle classification (dominant or subordinate follicle) and prenatal treatment (control, testosterone propionate (TP) and 5α-dihydrotestosterone (DHT)) for granulosa cell progesterone production over a six day serum-free culture. Values presented are the mean ± SEM. Follicle classification - 1 = DF, 2 = SF. Treatment classification – 1 = control, 2 = TP and 3 = DHT.

Granulosa Cell Progesterone Production – Data normalised to 1,000 live granulosa cells counted after 6 days of culture

Prenatal androgenisation by either TP or DHT had no overall effect on granulosa cell progesterone production at 144hrs by live cells from a smaller subset of DF and SF (Figure 6.12). Gonadotrophin addition to the media also did not effect granulosa cell progesterone production. There was no difference in granulosa cell progesterone production between DF and SF. Progesterone production from granulosa cells of DF and SF recovered from TP-treated ewes tended to be more similar than progesterone production by cells from DF and SF recovered from the control and DHT groups (P = 0.089). There was no interaction between the factors culture media and prenatal treatment; therefore, differences in gonadotrophin receptor mRNA expression in TP cells did not translate into a different progesterone response to gonadotrophins *in vitro*. The day that the granulosa cell culture was set-up had no effect on progesterone synthesis.

A



B

Comparisons of levels within factors	GLM P value
Day of culture 1 vs 2	0.534
Day of culture 1 vs 3	0.876
Day of culture 2 vs 3	0.432
Media – 0 vs FSH	0.552
Media – 0 vs LH&FSH	0.243
Media - FSH vs FSH&LH	0.367
Prenatal Treatment – Control vs TP	0.189
Prenatal Treatment – Control vs DHT	0.457
Prenatal Treatment – TP vs DHT	0.143
DF vs SF	0.450
Media*Prenatal Treatment	0.673
Prenatal Treatment and Follicle Classification Interaction	0.089

Figure 6.12 (A) Mean granulosa cell progesterone production from 96 to 144 hours of culture for cells cultured serum-free for 6 days and harvested from follicles classified as either dominant (DF) or subordinate (SF), recovered from prenatally androgenised (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) and control ewes. Production is combined across all day of culture set-ups and media conditions. Values presented are means ± SEM (B) P values obtained following GLM analysis when levels were compared within a factor. Day of culture refers to the start date of three independent cultures, (1 = day 1, 2 = day 2 and 3 = day 3), 0 media = no gonadotrophin supplement, FSH = 1 ng FSH supplemented media, FSH&LH = 1 ng FSH & 11.67 ng LH supplemented media. GLM = general linear model. DF – C = 5, TP = 7, DHT = 4; SF – C = 3, TP = 5, DHT = 3 follicles.

Percent change in Granulosa Cell Progesterone Production with addition of gonadotrophins to the media

The addition of LH to FSH had no significant effect on the percent change in granulosa cell progesterone production (relative to zero supplemented medium) in any of the treatment groups at 48 and 96hrs of culture (Figure 6.13). However, relative progesterone production was significantly increased after 144hrs of culture when LH was added to FSH, but only in the TP androgenised group (Figure 6.13).

6.3.4 Control (Abattoir) Granulosa Cell Cultures

Granulosa cell viability

The effect of the gonadotrophins (FSH and FSH&LH), time (48, 96 and 144hrs) and cell density plated (32,000, 16,000, 8,000 and 4,000 live cells plated) on absolute live granulosa cell numbers and percentage viability over a 6 day culture period is shown in Figure 6.14. The addition of FSH or LH together with FSH to the culture media had no effect on granulosa cell percentage viability and live cell numbers. From 0-48hrs granulosa cell percentage viability and live cells numbers significantly decreased ($P < 0.001$), however, from 48hrs to the end of the culture at 144hrs no significant difference in granulosa cell percentage viability and live cell numbers was determined. Higher cell densities improved the granulosa cell percentage viability and live cell number ($P < 0.001$). The number of live granulosa cells and granulosa cell percentage viability was reduced when cells were plated at 8,000 and 4,000 live cells in comparison to when cells were plated at 32,000 live cells ($P = 0.004$ and < 0.001, respectively). Granulosa cell percentage viability and live cell numbers over the six day culture period was also reduced when cells were plated at 4,000 compared to 16,000 live cells per well ($P < 0.001$). There was a tendency for a reduction in granulosa cell percentage viability and live cell numbers when cells were plated at 8,000 compared to 16,000 ($P = 0.067$). There was no difference in live cell numbers and granulosa cell percentage viability when cells were plated at 8,000 compared to 4,000 and between 32,000 and 16,000 live granulosa cells per well.

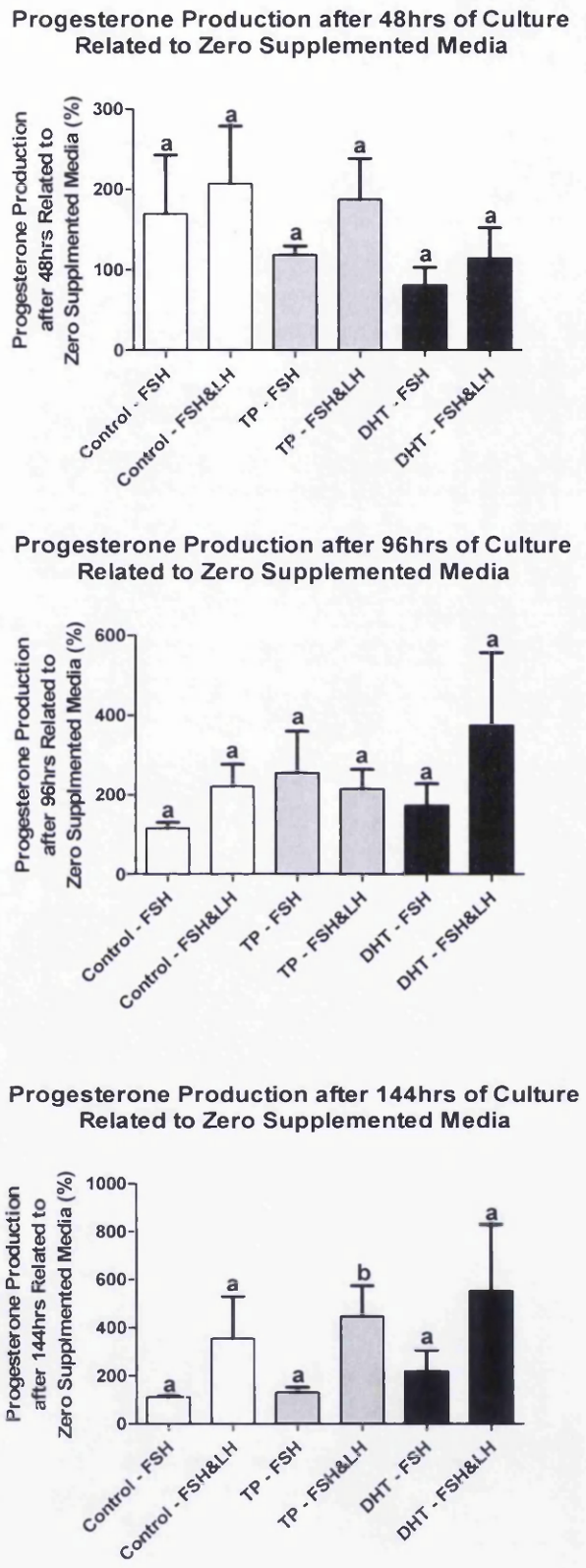
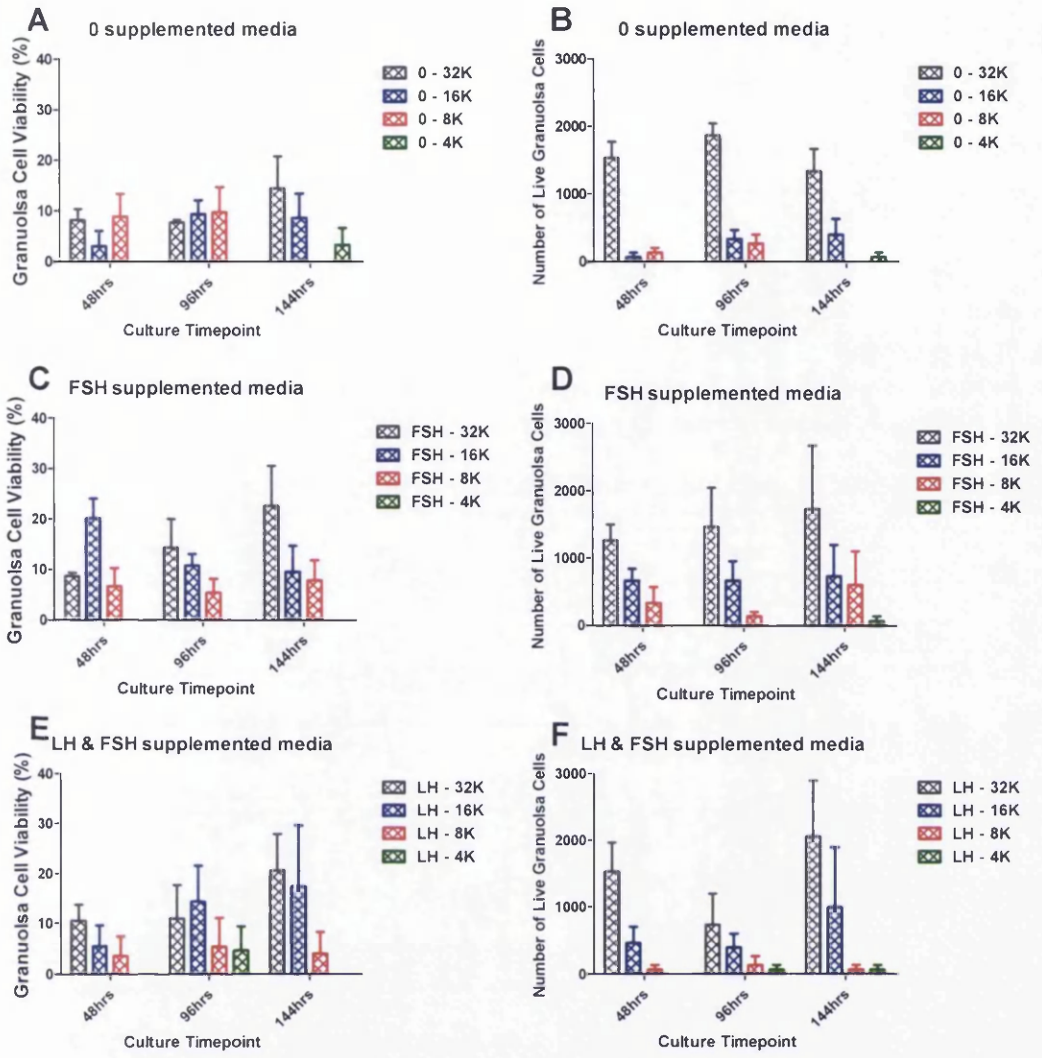


Figure 6.13 Effect of LH addition to FSH in media on the percent change in granulosa cell progesterone production *in vitro* after 48hrs (top panel), 96hrs (middle panel) and 144hrs (top panel) of culture, where follicles were recovered from prenatally androgenised (TP = testosterone propionate, DHT = 5α-dihydrotestosterone) and control ewes. Granulosa cell progesterone production is related to the production determined in zero supplemented media (no gonadotrophin). Values presented are means ± SEM. [a,b.] Within a treatment group, means without a common superscript differ ($P < 0.05$). Control = 11, TP = 14, DHT = 6 follicles.



Factor	GLM P Value - Percent Viability (Live cell number)	Comparisons of levels within a factor	Tukey Post-Hoc test P value - Percent Viability (Live cell number)
Culture Media	0.296 (0.245)	N/A	N/A
Culture Time-point	0.374 (0.236)	N/A	N/A
Cell Density	0.000 (0.000)	32K vs 16K	0.732 (0.435)
		32K vs 8K	0.004 (0.001)
		32K vs 4K	0.000 (0.004)
		16K vs 8K	0.067 (0.089)
		16K vs 4K	0.000 (0.003)
		8K vs 4K	0.112 (0.334)

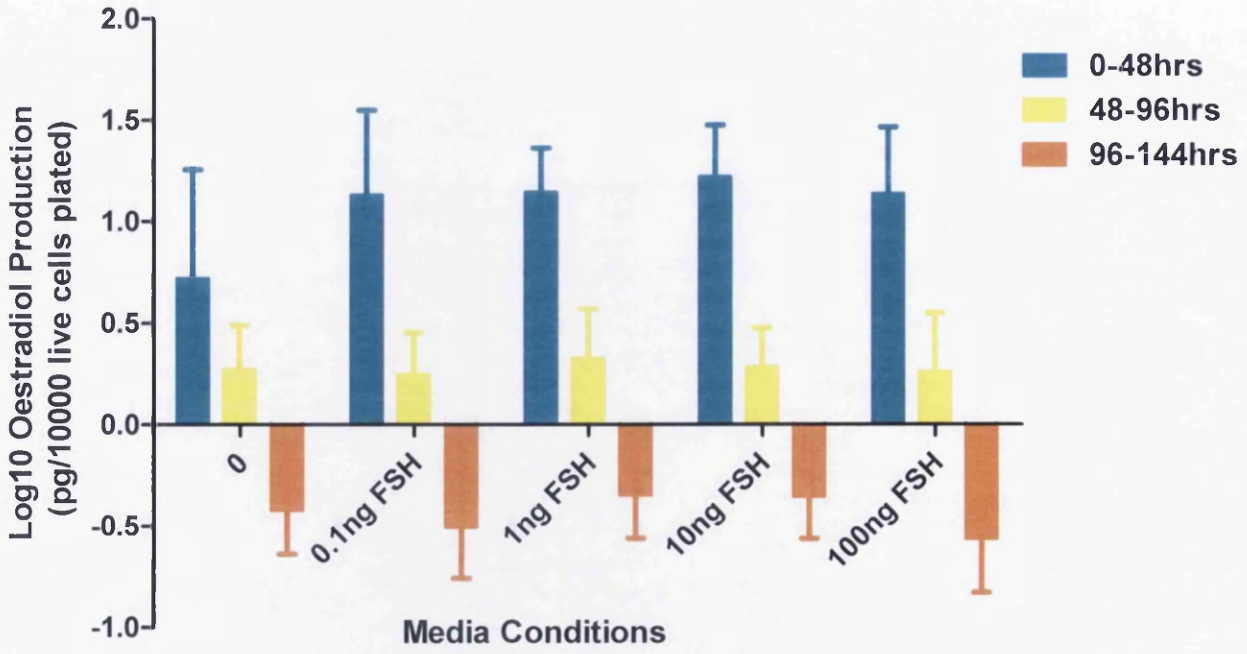
Figure 6.14 Mean percentage granulosa cell viability (A, C, E) and absolute granulosa live cell number per well (B, D, F) at three time-points (48, 96 and 144hrs) over a 6 day serum free culture system (n = 3 independent cultures). P values following Generalised Linear Model (GLM) analysis for each factor (and, where appropriate, levels within a factor) are shown in the table below the graphs, where percentage viability was used as the outcome measure. The values in parentheses are the P values obtained when granulosa live cell number was used as the outcome measure. Values presented are mean ± SEM. 0 – zero supplemented media, FSH – 1 ng oFSH supplemented media, LH – 11.67 human recombinant LH supplemented media. 32K – 32,000 live granulosa cells plated, 16K – 16,000 live granulosa cells plated, 8K – 8,000 live granulosa cells plated, 4K – 4,000 live granulosa cells plated.

Responses to oFSH

The effect of varying concentrations of ovine FSH on granulosa cell oestradiol and progesterone production over a 6 day culture period is shown in Figures 6.15 and 6.16 respectively. We were unable to demonstrate that cultured granulosa cells from large (>5 mm) ovine follicles exhibited a dose-dependent oestrogenic response to FSH. Although absolute levels of progesterone production were elevated when higher doses of oFSH were used, we were unable to determine a significant dose-dependent progesterone response to oFSH. Similar to the experimental culture (granulosa cells from control and androgenised ewes), granulosa cell oestradiol production per live cell plated dramatically reduced during the culture period, while progesterone production increased throughout the 6 day culture ($P < 0.001$).

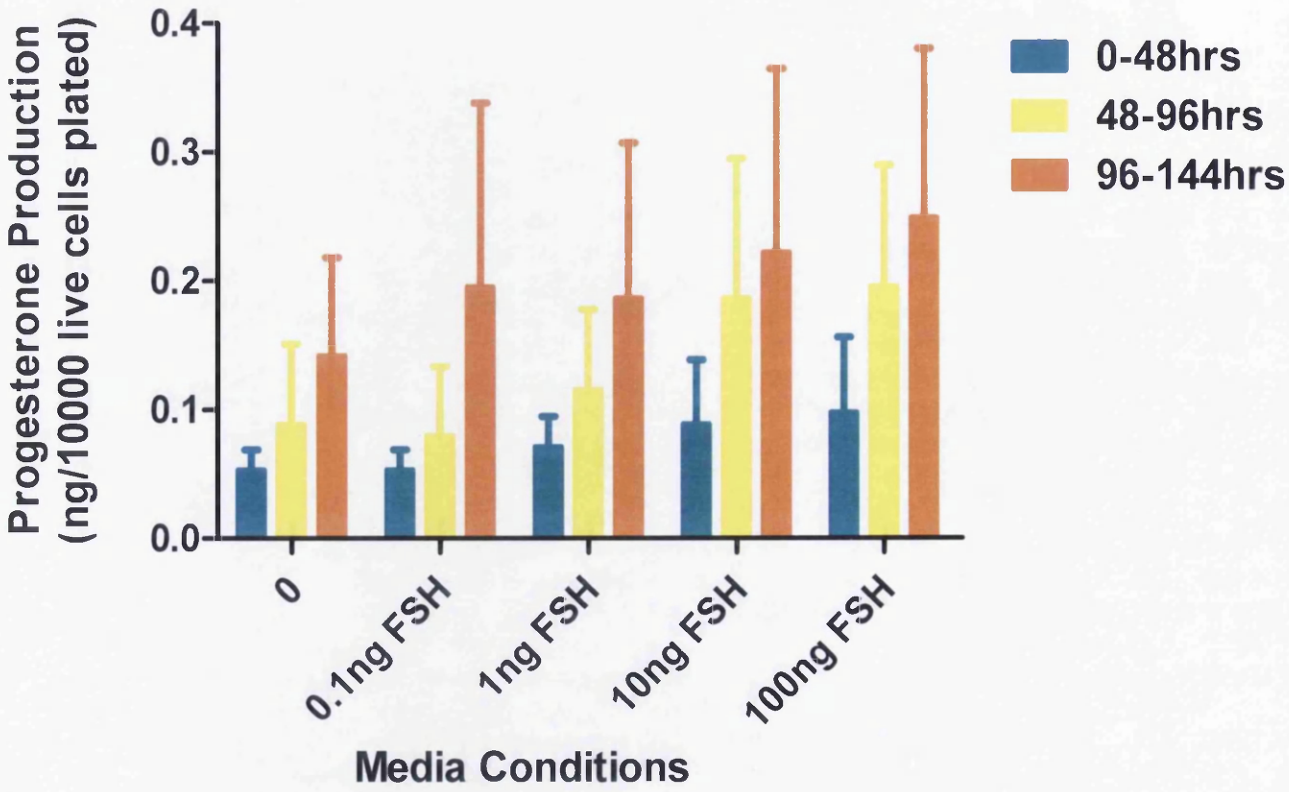
Responses to oLH

The effect of varying concentrations of ovine LH on granulosa cell oestradiol and progesterone production over a 6 day culture period is shown in Figures 6.17 and 6.18, respectively. Human recombinant LH was also included as this was the exogenous LH source used in the experimental granulosa cell culture and it was necessary to compare its biological activity with that of ovine LH. We were unable to demonstrate that cultured granulosa cells from large (>5 mm) ovine follicles exhibited a dose-dependent oestrogenic or progestagenic response to exogenous ovine LH (when normalised to cells plated). Although absolute levels of progesterone produced were elevated using higher doses of oLH and the dose of recombinant human LH used, we were unable to determine a significant progesterone response. Similar to the experimental culture (granulosa cells from control and androgenised ewes) granulosa cell oestradiol production (normalised to 10,000 cells plated) was reduced during the culture period ($P = 0.04$) partially reflecting the loss of cells over the first 48h, while progesterone production tended to increase throughout the 6 day culture period ($P = 0.08$) indicating an increase in progesterone production per cell. The human recombinant LH dose produced an absolute progesterone response similar to that when an exogenous dose of 100 ng of oLH was used.



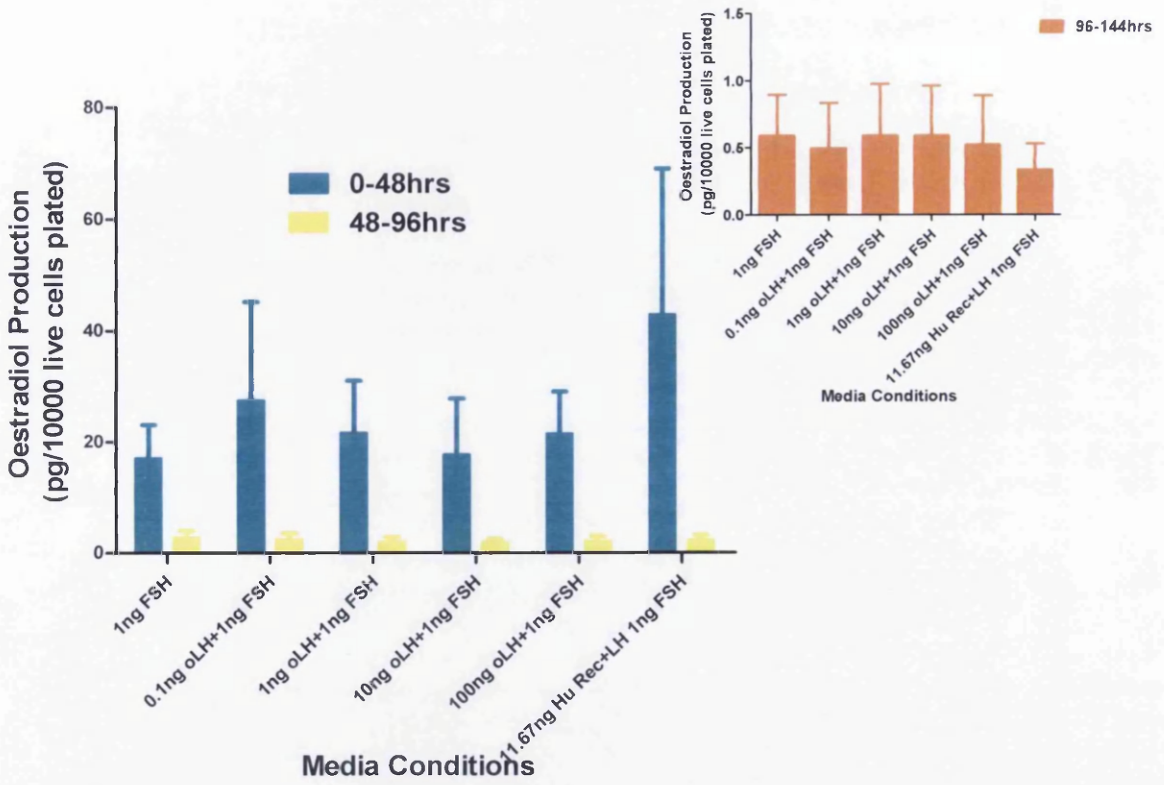
Factor	GLM P Value	Comparisons of levels within a factor	Tukey Post-Hoc test P value
Culture Media	0.909	N/A	N/A
Culture Time-point	0.000	48 vs 96hrs	0.001
		48 vs 144hrs	0.001
		96 vs 144hrs	0.952
Culture Media*Culture Time-point	0.972	N/A	N/A

Figure 6.15 Effect of ovine follicle stimulating hormone (oFSH) on mean granulosa cell oestradiol production after culture for 6 days under serum-free conditions. Granulosa cells were recovered from follicles ≥5 mm in diameter from slaughterhouse ovaries when animals were out of the breeding season. Conditioned media was harvested and replaced with fresh media every 48 hours of culture. Values presented are means ± SEM of three independent replicate cultures. N/A = not applicable.



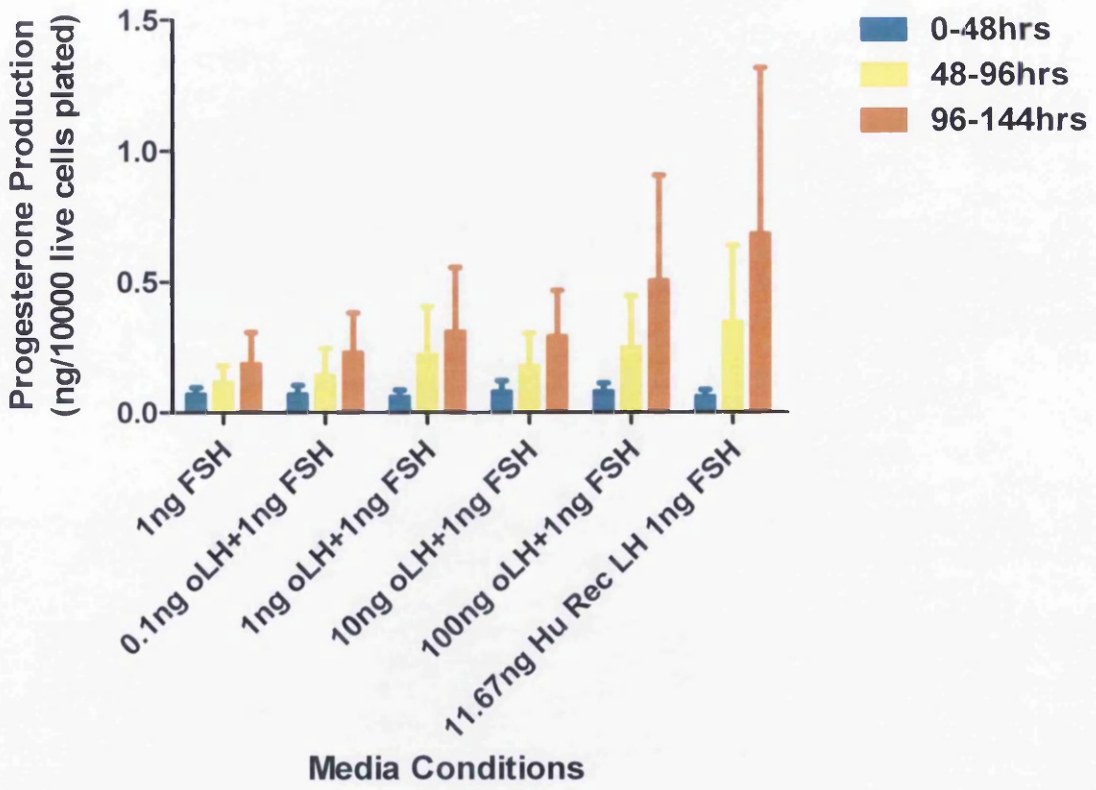
Factor	GLM P Value	Comparisons of levels within a factor	Tukey Post-Hoc test P value
Culture Media	0.826	N/A	N/A
Culture Time-point	0.000	48 vs 96hrs	0.001
		48 vs 144hrs	0.001
		96 vs 144hrs	1.000
Culture Media*Culture Time-point	0.919	N/A	N/A

Figure 6.16 Effect of ovine follicle stimulating hormone (oFSH) on mean granulosa cell progesterone production after 6 days of culture under serum-free conditions. Granulosa cells were recovered from follicles ≥5 mm in diameter from slaughterhouse ovaries when animals were out of the breeding season. Conditioned media was harvested and replaced with fresh media every 48 hours of culture. Values presented are means ± SEM of three independent replicate cultures. N/A = not applicable.



Factor	GLM P Value	Comparisons of levels within a factor	Tukey Post-Hoc test P value
Culture Media	0.721	N/A	N/A
Culture Time-point	0.037	48 v 96hrs	0.873
		48 vs 144hrs	0.037
		96 vs 144hrs	0.753
Culture Media*Culture Time-point	1.000	N/A	N/A

Figure 6.17 Effect of ovine luteinising hormone (oLH) on mean granulosa cell oestradiol production during 6 days of culture under serum-free conditions. Granulosa cells were recovered from follicles ≥5 mm in diameter from slaughterhouse ovaries when animals were out of the breeding season. Conditioned media was harvested and replaced with fresh media every 48 hours of culture. Note, a dose of 11.67 ng human recombinant luteinising hormone (LH) was also included as this was the source of exogenous LH used in the experimental granulosa cell culture. Values presented are means ± SEM of three independent replicate cultures. FSH = follicle stimulating hormone; N/A = not applicable. Note that the vertical scale bar for 96-144hrs steroid production is different from that for -48hrs and 48-96hrs.



Factor	GLM P Value	Comparisons of levels within a factor	Tukey Post-Hoc test P value
Culture Media	0.779	N/A	N/A
Culture Time-point	0.081	48 v 96hrs	0.866
		48 vs 144hrs	0.079
		96 vs 144hrs	0.666
Culture Media*Culture Time-point	0.996	N/A	N/A

Figure 6.18 Effect of ovine luteinising hormone (oLH) on mean granulosa cell progesterone production during 6 days of culture under serum-free conditions. Granulosa cells were recovered from follicles ≥5 mm in diameter from slaughterhouse ovaries when animals were out of the breeding season. Conditioned media was harvested and replaced with fresh media every 48 hours of culture. Note, a dose of 11.67 ng human recombinant LH was also included as this was the source of exogenous LH used in the experimental granulosa cell culture. Values are means ± SEM of three independent replicate cultures. FSH = follicle stimulating hormone; N/A = not applicable.

6.4 DISCUSSION

The results in this study provide evidence for the first time that prenatal androgenisation by DHT reduces inherent (gonadotrophin-independent) oestradiol production by granulosa cells from the largest two follicles *in vitro* using a serum-free culture system which provides the hormones insulin, IGF-1, and the oestrogen precursor androstenedione, with and without FSH and LH. Prenatal androgenisation by DHT also reduced progesterone production by granulosa cells *in vitro*, but, in accordance with the *in vivo* follicular fluid results, only relative to cells recovered from TP-follicles. Thus, the androgenic action of prenatal testosterone decreases granulosa cell steroid production from DF and SF *in vitro* in adulthood. Prenatal androgenisation by TP had no overall significant effect on granulosa cell oestradiol production relative to controls, but appeared to affect the oestrogenic response to FSH *in vitro*, tended to increase *in vitro* progesterone production overall, and showed a significant progestagenic response to LH addition to FSH in the culture medium. However, the lack of a significant media with treatment interaction in the analyses of *in vitro* oestradiol and progesterone production seems to indicate that granulosa cells from androgenised ewes do not show very large differences to controls in the steroidogenic response to FSH and LH. Thus, either granulosa cells from control animals are more responsive to the culture conditions (e.g. where excess insulin or IGF-1 are provided) than under *in vivo* conditions, or an important growth factor/hormone is not supplied within our culture system which exists within the peripheral circulation of TP-treated ewes. It is important to bear in mind, that in control setups the dose and type of LH used also did not significantly enhance *in vitro* oestradiol or progesterone production. Thus, lack of differential response to LH may be due to the lack of biopotency of the LH used. However, control setups were performed one month later in May (non-breeding season), where granulosa cells obtained from anovulatory large follicles may perhaps have altered LH responsiveness compared with ovulatory follicles. These findings together with discussion of the granulosa cell culture system used in the study are described in further detail below.

Data Normalisation Methods

In order to discuss the results from this study in greater detail it is important to highlight that for granulosa cell steroid production several data normalisation methods were used (i.e. steroid production per 1,000 live cells plated, steroid production per 1,000 live cells counted after 6 days of culture, percent change relative to production at another time-point or under a different media condition). Previous *in vitro* studies investigating steroid production over 6 days, normalise production between 96-144hrs to the number of live cells at the end of culture (Campbell *et al.*, 1996; Campbell *et al.*, 2006). Steroid

production between 96 and 144 hours is chosen as this period represents the physiological responsiveness of recovered granulosa cells (Armstrong *et al.*, 1996; Smith *et al.*, 2005; Silva *et al.*, 2006). However, due to so few live granulosa cells detected after 6 days of culture using this culture system (probably as a result of so few cells plated) other data normalisation methods such as those listed above were implemented. Such data normalisation has been used successfully in older publications, for bovine cells from preovulatory follicles (Fortune, 2003), bovine cells from antral follicle 1-8 mm in diameter (Langhout *et al.*, 1991) and rat cells from antral follicles (Fortune & Vincent, 1983). As a result, there are some examples where the results from the two data normalisation methods do not correlate (e.g. follicle classification and granulosa cell progesterone production – significant difference between DF and SF when steroid production normalised to 1,000 live cells plated, however this significance is lost when steroid production is related to 1,000 live cells after 6 days of culture). Such differences may also be due to only a small subset of samples being available for analysis of steroid production related to live cells counted at 144h; with the inherent large variation of culture setups, differences may not become apparent until a larger sample set is used. Any differences in results when steroid production was normalised using the different methods will be highlighted.

Androgenic programming of granulosa cell steroid production

Prenatal androgenisation by DHT but not TP reduced granulosa cell oestradiol production overall (data normalised to 1,000 live cells plated). Additionally, progesterone production by cells from DHT-treated animals decreased when compared to cells from TP-treated animals (data normalised to live cells plated), but was similar compared to controls. Therefore, the androgenic action of testosterone administered *in utero* reduces the ability of granulosa cells recovered from adult ewes to produce oestradiol and progesterone in the *in vitro* environment used for this study. The decrease in oestradiol and progesterone production may be due to an intrinsic lowered synthesizing capacity, as granulosa cells of both DF and SF from DHT-treated ewes were affected, independent of gonadotrophin stimulation during *in vitro* culture. Androgenisation by DHT reducing granulosa cell oestradiol and progesterone steroid production in comparison to cells from TP-treated animals replicates the *in vivo* results determined in **Chapter 3**, where follicular fluid oestradiol and progesterone concentrations within DF and SF also were lower in follicles from DHT versus TP-treated ewes. Taken together, these results add weight to the theory that it is the oestrogenic action of testosterone that programmes postnatal follicular events such that it drives increased steroid production from large antral follicles, and suggests that the androgenic action of testosterone if anything may actually cause an intrinsic reduction

of the ability of granulosa cells to produce steroids. The underlying mechanism that leads to a reduction in granulosa cell steroid production *in vitro*, particularly in relation to LH responsiveness should be investigated, given hypersecretion of LH present in DHT-treated ewes (Veiga-Lopez *et al.*, 2009). In light of this, future studies should investigate granulosa cell expression profiles of *LHR*, *FSHR*, *CP19A1* and *HSD3B1* in granulosa cells recovered from DHT-treated ewes.

This study aimed to address whether increased steroidogenesis observed in DF and SF from prenatally TP-treated ewes (**Chapter 3**) is as a result of an intrinsic increase in granulosa cell steroidogenesis or if granulosa cells have perhaps an increased functional response to LH and reduced response to FSH. If prenatal androgenisation by TP results in an intrinsic alteration in granulosa cell steroidogenesis, we would expect granulosa cell steroid production to be increased when cells were cultured in the same media (e.g. zero supplemented media). If however, an altered response to either gonadotrophin is present within granulosa cells (i.e. LH stimulation would lead to an enhanced, and FSH stimulation to reduced granulosa cell oestradiol and/or progesterone production *in vitro*) we would expect a significant interaction between prenatal treatment and media hormone supplementation.

Differential Response to Gonadotrophins

No media with prenatal treatment interaction for both oestradiol and progesterone production was determined in this study (when data were normalised using both 1,000 live cells plated and 1,000 live cells after 6 days of culture). Therefore, differences in gonadotrophin receptor mRNA expression in TP cells determined in **Chapter 5** did not translate into a differential response to gonadotrophins *in vitro*. However, within granulosa cells of this study, prenatal androgenisation by TP reduced granulosa cell *FSHR* exon 9/10 expression, while *LHR* expression was similar between treatment groups. Therefore, despite prenatal treatment likely increasing the expression and thus the amount (number) of LHR present in granulosa cells of DF and SF (Ireland & Roche, 1983b) not enough potent LH was possibly available in the cell culture system to lead to a very strong differential response (either increase in oestradiol or progesterone production). In absolute terms, the recombinant human LH used clearly was more potent than even the highest dose of ovine LH. This is not the case for FSHR, where the FSH dose given was sufficient to increase oestradiol (but not progesterone) granulosa cell production. Therefore, the dose of FSH used in the experimental culture was biopotent. *In vitro* results not replicating those seen *in vivo* (i.e. the follicular fluid differences) could be explained as a result of the presence of

potent growth factors within our culture system (e.g. insulin and IGF1, a type which is not neutralised by IGF binding proteins) (Ryan *et al.*, 2008), not normally present in such concentrations *in vivo*. If they are present in too high a concentration *in vitro*, they may override any major reduction in granulosa cell steroid production in response to FSH. Both insulin and IGF-1 have been shown to increase ovine granulosa cell oestradiol and progesterone production *in vitro* from both small and large antral follicles (Campbell et al., 1996). In addition, FSH has been shown to enhance granulosa cell responsiveness to IGF-1 (Glister *et al.*, 2001), rather than the opposite, leading to the possibility of high doses of IGF masking any effects of FSH. However, there was some evidence of a differential response to FSH in terms of granulosa cell oestradiol production when a different data normalisation method was implemented. Granulosa cells obtained from DF and SF from control ewes showed a larger percent increase in oestradiol production when FSH was added to the zero supplemented media compared with cells from TP- and DHT-treated ewes. Thus, using this method of normalisation prenatal androgenisation by TP and DHT appears to reduce granulosa cell FSH responsiveness to some extent. This *in vitro* result matches those seen *in vivo* in chapter 4 and results from granulosa cells in this study, where *FSHR* mRNA expression is downregulated in granulosa cells of DF and SF as a result of TP prenatal androgenisation.

DF and SF granulosa cells recovered from prenatally TP androgenised ewes did not respond to LH differentially in either oestrogen or progesterone production compared with cells obtained from follicles of control ewes (data normalised to 1,000 live cells plated and 1,000 live cells after 6 days of culture), because of lack of a significant interaction. However, there was some evidence of an enhanced LH response in cells from androgenised ewes: when the percent change in oestradiol production from 96 to 144 hours was evaluated in media supplemented with FSH & LH, androgenisation led to maintenance of oestradiol production compared to a severe drop during culture of granulosa cells recovered from control ewes. In addition, the percent change in progesterone production at 144 hours following LH addition to FSH was only significant when cells were cultured from TP androgenised ewes. Thus, this hints at a slightly increased responsiveness to LH in granulosa cells of DF and SF from TP-treated ewes. Increased responsiveness to LH agrees with results seen *in vivo* of increased *LHR* mRNA expression in granulosa cells of DF and SF recovered from TP-treated ewes (**Chapter 5**). Despite an indication that DF have higher LHR mRNA in TP cells used for this *in vitro* study, this did not translate into a higher functional steroid response to LH *in vitro*.

Gonadotrophin Independent Granulosa Cell Steroid Production

The lack of a significant prenatal treatment and culture media interaction on both granulosa cell oestradiol and progesterone production suggests steroid production is altered independent of gonadotrophin stimulation. In fact, a low overall tendency for increased progesterone production in cells from TP androgenised ewes was found. In addition, when the percent change in granulosa cell oestradiol production from 96 to 144 hours was evaluated in zero supplemented, androgenisation led to a maintenance in oestradiol production, compared with a severe drop during culture of cells from control ewes. Thus, this finding is consistent with the belief that it is not the peripheral gonadotrophin environment that results in increased steroidogenesis within DF and SF (**Chapter 3**), but that prenatal androgenisation by TP programs increased granulosa cell steroidogenesis within DF and SF. Prenatal androgenisation programming increased androgenisation could be achieved either by changing other hormonal mediators of steroid synthesis not yet evaluated, such as insulin and IGF-1 (Spicer *et al.*, 1993; Glister *et al.*, 2001), or by altering the inherent constitutive capacity of granulosa cells from TP androgenised ewes for oestradiol and progesterone synthesis.

Follicle classification and hierarchy

Our follicle classification criteria was given further validation as follicles classified as DF over all three treatment groups produced more oestradiol *in vitro* than those classified as subordinate, which is indicative of a dominant follicle (Evans & Fortune, 1997; Ireland *et al.*, 2004). Increased granulosa cell oestradiol production concurs with elevated oestradiol concentrations found within the follicular fluid of DF in the third chapter. Granulosa cells from follicles classified as SF produced more progesterone than those classified as DF, thus mimicking progesterone concentrations within the follicular fluid of SF and DF found in other studies (Evans & Martin, 2000). This is in contrast to the intrafollicular progesterone concentrations from the animals used in this study, where concentrations were similar in SF compared to DF. Again, the disparity between the *in vivo* and *in vitro* results could be as a result of the *in vitro* environment not mimicking that seen *in vivo*, with a growth factor or hormones (perhaps GH, insulin, IGF-1) either absent or in higher than normal concentrations. Progesterone production from DF and SF *in vitro* was similar when recovered from prenatally androgenised ewes from TP only, whereas production increased in SF compared to DF in both the control group and DHT-treated group. Oestradiol production in DF and SF classified follicles from TP-treated animals was also similar, in comparison to those recovered from control and DHT-treated ewes, where oestradiol production was significantly higher in DF compared to SF. This confirms results

observed in **Chapter 3** and **Chapter 5** of prenatal TP treatment leading to increased steroidogenesis (oestradiol and progesterone) within the largest antral follicles, and suggests that prenatal androgenisation by TP results in the loss of functional follicle hierarchy. To the best of our knowledge no group has investigated steroid production within dominant and subordinate follicles recovered from prenatally androgenised ewes *in vitro*. Therefore, for the first time these results provide *in vitro* evidence that follicle hierarchy is diminished as a result of prenatal androgenisation.

Culture System Used

Within our experimental culture system, FSH stimulated the production of oestradiol; however, the addition of 11.67 ng human recombinant LH did not further enhance oestradiol production. In fact, the addition of LH to FSH reduced such enhancement of oestradiol production. Ovine FSH stimulation of oestradiol production in the experimental cultures is in contrast to our dose response control setup, where we were unable to demonstrate a dose oestradiol response to 1 ng FSH. This could be as a result of the data normalisation method used for the dose response culture, as results were normalised to 10000 live cells plated. Additionally, in our experimental culture, GLS analysis showed that a 11.67 ng dose of human recombinant LH increased progesterone granulosa cell production when data were normalised to live cells plated. We were unable to demonstrate a dose response to oLH on oestradiol or progesterone production in our control culture, although in absolute values, higher doses of LH stimulated increased production of progesterone. As mentioned above, the effect of a 1 ng dose of FSH on oestradiol production differed between the experimental culture and the dose-response culture. This difference between the two cultures could be as a result of follicles being recovered at different stages of follicle development. In the experimental study, only DF and SF follicles were recovered (some of which were less than 5 mm in control animals, thus possibly including growing FSH dependent follicles (Dufour *et al.*, 1979; Miller *et al.*, 1979), whereas the granulosa cells from follicles >5 mm in diameter were recovered for the dose response culture. Within this group older, regressing non-ovulatory follicles may be included, which are unresponsive to both FSH and LH in terms of cAMP production (Jolly *et al.*, 1997a). The reasons for a lack of response to exogenous LH on granulosa cell oestradiol and progesterone production are difficult to determine given that we followed a previously published and well characterised culture system (Gutierrez *et al.*, 1997; Glister *et al.*, 2005).

General Steroid Production

Granulosa cell oestradiol production reduced over time (when normalised to 1,000 live cells plated) within our 6 day culture of experimental cells plated at very low densities (at all time points), in concordance with our dose response control culture where high starting cell densities.could be employed. In contrast, during experimental cultures progesterone production normalised to 1,000 live cells plated was maintained using GLM analysis (in fact it was significantly increased when analysed using GLS) despite a clear loss of live granulosa cells. Therefore, despite using a well characterised culture system that is able to respond to FSH with physiological concentrations of oestradiol (Gutierrez *et al.*, 1997; Glister *et al.*, 2005), and having shown an FSH effect on oestradiol secretion in this study, the overall steroid secretion patterns observed suggest that this culture system promoted luteinisation of granulosa cells.

As it was only possible to culture a very small number of granulosa cells per well within the experimental culture, and cells are lost during culture, this resulted in the difficulty to estimate live cell numbers at 144h for normalising steroid results. As any differences in steroid production per well (or normalised to the number of cells plated) could be due to a differential decline in live cell numbers as a result of different sources of cells and added hormones, an investigation into the rate of granulosa cell death and the effect cell density had on subsequent granulosa cell survival and viability was instigated.

The number of live granulosa cells plated was similar per treatment group per follicle classification. This is important to determine as plating density (of bovine granulosa cells) has been shown to alter steroid production and cell health, albeit this was determined when a very large number of cells (250,000 to 1.5 million live granulosa cells per well) were plated (Portela *et al.*, 2010). Low plating density favours oestradiol secretion and mRNA encoding estrogenic enzymes, whereas higher density inhibites oestradiol production and enhances progesterone secretion and levels of mRNA encoding progestagenic enzymes (Portela *et al.*, 2010). Increasing plating density decreased the oestrogen: progesterone ratio and reduced cell health (Portela *et al.*, 2010). Our study also revealed that cell density had a significant effect on granulosa cell survival and viability. However, cell density only had significant effects on granulosa cell viability and live cell numbers when cells were plated at 32,000 and 16,000 compared to 4,000 live cells per well. Importantly, there was no significant difference when granulosa cells were plated at 8,000 and 4,000. The average number of live granulosa cells plated was 3205, 7337 and 3917 for DF from the control, TP and DHT treatment groups, respectively, and 1822, 6384 and 2256 live granulosa cells

for SF from control, TP and DHT groups, respectively. Therefore, the granulosa cell density at which follicles were plated is not expected to have an effect on granulosa cell live cell numbers and viability over the 6 day culture period. Therefore, from this observation it is likely that the rate of granulosa cell death is similar between these plating densities and thus across all three treatment groups. Of note is the fact that the number of live granulosa cells significantly dropped dramatically during 48 hours of culture, independent of the densities the granulosa cells were plated at. Live granulosa cells were not lost during the process of replacing conditioned media with fresh media, as no live granulosa cells were present in the conditioned media (data not shown). Therefore, it is unknown why using a well established culture system (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997; Glister *et al.*, 2005) resulted in such a dramatic loss of live granulosa cells during the initial 48 hours of culture.

To the best of our knowledge, no other attempts have been made to culture granulosa cells from individual ovine follicles for a period of 6 days. As previously stated, granulosa cells were cultured for six days as past studies have determined that only the 96-144h time period appears to reflect the physiological responsiveness of recovered granulosa cells (Armstrong *et al.*, 1996; Smith *et al.*, 2005; Silva *et al.*, 2006). As the number of live granulosa cells plated was so low, it is hard to directly compare the results from our culture system to others. Previous granulosa cell cultures have varied in terms of source of the granulosa cells (emerging follicles, dominant follicles etc.) (Meidan *et al.*, 1992; Spicer *et al.*, 1993; Glister *et al.*, 2005; Campbell *et al.*, 2006; Ryan *et al.*, 2008), culture conditions and added hormones, whether cells from dissected follicles were cultured independently or pooled, the density granulosa cells were plated at, the model organism used (sheep or cattle) and, finally, the duration of culture (Jimenez-Krassel & Ireland, 2002; Jimenez-Krassel *et al.*, 2003; Ireland *et al.*, 2004; Campbell *et al.*, 2006). Typically, culture periods have been of either a shorter duration or identical to ours ranging from a culture period of 18hrs (Jimenez-Krassel & Ireland, 2002; Jimenez-Krassel *et al.*, 2003; Ireland *et al.*, 2004; Campbell *et al.*, 2006) to 144hrs (Gutierrez *et al.*, 1997; Ryan *et al.*, 2008). Additionally, cultures have pooled follicles in order to achieve the optimum cell density for proliferation and oestradiol production of the granulosa cells, thought to be between 50,000 and 75,000 cells per well (Gutierrez *et al.*, 1997; Jimenez-Krassel & Ireland, 2002). One group cultured granulosa cells from individual DF and SF from cattle (Ireland *et al.*, 2004), where DF are much larger in size than those found in sheep, enabling enough live granulosa cells to be recovered to achieve the desired density. In our study, we chose not to pool granulosa cells and to culture granulosa cells from individual follicles, as we wanted

to gain additional information on the follicle hierarchy within androgenised ewes. If follicles were pooled this information would be lost.

Gene expression profiles following *in vitro* culture

Unfortunately, investigation into the mRNA expression profiles of FSH- and LH-responsive genes in granulosa cells after culture, which may have confirmed differences in survival or luteinization gene expression as well as in gonadotrophin responsiveness, was not possible, due to the low numbers of live granulosa cells plated. Thus, with the inevitable cell loss during the 6-day culture, we were unable to recover adequate amounts of RNA from cells after culture, even when a specialised RNA extraction kit was utilised to isolate high quality and quantity RNA from very small samples. Therefore, future studies should pool the granulosa cells collected from several follicles of prenatally androgenised ewes in order to determine the expression profiles of FSH- and LH-responsive genes following *in vitro* stimulation. As stated earlier, the pooling of follicles with the same classification has been used in previous granulosa cell culture studies due to the low number of live granulosa cells isolated and the variability in the numbers of granulosa cells isolated from individual follicles (Ireland *et al.*, 2004). Pooling of granulosa cells isolated from individual follicles would enable an increased number of live granulosa cells to be plated, comparable to that used in previous studies (75,000 – 100,000 live cells/200µl for culture in 96 well plates) (Ireland *et al.*, 2004; Campbell *et al.*, 2006). However, significantly more follicles would have to be recovered (therefore many more prenatally androgenised sheep would be required) if follicles with the same classification are to be pooled for *in vitro* cell culture. The results from such a study would not only help determine whether the differential gonadotrophin receptor expression between control and TP cells is maintained *in vitro*, thus help in elucidating whether the lack of a strong functional differential gonadotrophin responsiveness was due to equal receptor expression or the equally supportive *in vitro* conditions, but also provide direct functional evidence as to how the gonadotrophins, particularly LH, stimulate granulosa cell steroid production and enhanced growth *in vivo* (results in **Chapter 3** and **Chapter 5** suggest LH is the instigator of enhanced growth and increased steroidogenesis). Additionally, such a study would be an ideal approach to investigate the possible induction of other luteinisation genes, or other response systems (other growth factors) which may be altered before and after culture.

In conclusion, the results of this experiment have demonstrated for the first time the effects of prenatal androgenisation on granulosa cell steroid production *in vitro* and delineated the

relative action of androgens and oestrogens *in utero* on the programming of follicular steroid production in the early adult ewe. Androgenisation by DHT results in a, most likely, inherent reduction of the ability of granulosa cells recovered from DF and SF to synthesise oestradiol. Studies provided some functional evidence that prenatal programming by TP reduces FSH stimulated granulosa cell oestradiol production, but may enhance progesterone production, both unstimulated and following LH stimulation, in adult ewes. Prenatal androgenisation also results in the loss of follicle hierarchy *in vitro* in relation to follicular capacity for steroid synthesis.

The prenatal environment may be the origin of a number of diseases in later life, including coronary heart disease and the related disorders stroke, diabetes, and hypertension (Barker, 1997). Prenatal programming by intrinsic and extrinsic factors that impact on the foetus can also lead to growth, metabolic and reproductive alterations in the offspring, the effects of which are manifested during adulthood (Holemans *et al.*, 1998; Tchernitchin *et al.*, 1999; Eriksson & Forsen, 2002; Walker & Humphries, 2007). Research from a wide range of scientific disciplines has shown that the reproductive performance of animals in adult life is also determined, in part, by a variety of environmental and nutritional influences acting at different stages of development from before conception until after birth (Rhind *et al.*, 2001). Thus, in addition to being of fundamental scientific interest, results from this research will provide further information on the effects of excess testosterone *in utero* on the programming of antral follicle function.

The primary aim of this thesis was to determine the effects of prenatal testosterone treatment on ovarian follicle development, specifically on terminal follicle development by investigating antral follicle growth, steroidogenesis, apoptosis and differentiation using a variety of methods. Given that the majority of previous studies have used the Suffolk breed of sheep, which is less sensitive to the effects of prenatal androgens, and have focused on neuroendocrine defects (Sharma *et al.*, 2002; Sarma *et al.*, 2005; Manikkam *et al.*, 2008; Veiga-Lopez *et al.*, 2008; Jackson *et al.*, 2009), follicle recruitment within the ovary (Steckler *et al.*, 2005; Smith *et al.*, 2008) and large antral follicle growth (Manikkam *et al.*, 2006; Steckler *et al.*, 2007a), there was clearly a requirement to investigate the effects of prenatal androgens on the programming of antral follicle function and differentiation, especially when one considers the abnormal multifolliculate morphology observed in these animals (West *et al.*, 2001; Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). Thus, the molecular aspects of ovarian function that can be programmed by excess prenatal androgens would be identified and investigation into the possible mechanisms that result in abnormal ovarian function, specifically on antral follicle dysfunction, would be possible.

This study into the effects of prenatal androgenisation on reproductive physiology is unique, as we have obtained data in several groups of sheep over five separate years (Clarke *et al.*, 1977; Birch *et al.*, 2003; Unsworth *et al.*, 2005; Manikkam *et al.*, 2006; Steckler *et al.*, 2007a). As a result, we are able to identify effects that are consistent over several years. Therefore, this enables those ovarian/follicle characteristics that are most likely to be programmed by excess androgen *in utero* to be identified and reduce the

possibility that the abnormalities isolated are as a result of a difference in maternal body condition or the external environment that both mothers and their offspring are exposed to throughout their lives (Luther *et al.*, 2007).

Another advantage of the present study was that a range of techniques were used to identify and characterise the effect of prenatal androgenisation on antral follicle function and development. Specifically, concentrations of follicular steroids (radioimmunoassay), gene expression (real-time PCR), protein localisation (immunocytochemistry) and follicle health (histology) were used to identify abnormalities in antral follicle development. As a result, a comprehensive investigation into the effects of excess testosterone *in utero* on antral follicle function was achieved.

Prenatal androgenisation by excess TP resulted in increased antral follicle growth and steroidogenesis through the oestrogenic action of testosterone (**Chapter 3**). The increase in systemic oestradiol, increased mRNA expression of *LHR* and *HSD3B1* and reduced mRNA expression of *FSHR* in follicular granulosa cells (**Chapter 5**) points to higher LH-dependent differentiation of large antral follicles from young androgenised ewes. Understanding the role LH has on the various aspects of reproduction dysfunction within the androgenised ewe will allow for a greater insight into the mechanisms underlying excessive antral follicle growth and increased steroidogenesis. Given that increased growth and enhanced steroidogenesis is achieved only when TP is used for prenatal androgenisation, and both prenatal treatment androgens (TP and DHT) result in hypersecretion of LH (Veiga-Lopez *et al.*, 2009), it appears that the LH response within follicles is abnormally activated in TP-treated ewes. GnRH antagonist treatment with gonadotrophin supplementation in the follicular phase in the prenatally androgenised (TP) Suffolk model (with a less severe phenotype), demonstrated that persistent large follicles are still able to respond to different LH environments with atresia or ovulation (Steckler *et al.*, 2008). This supports the concept of high LH responsiveness in large growing follicles from prenatally androgenised ewes. Therefore, we propose that the enhanced growth and steroidogenesis of several follicles is as a result of elevated LH. To answer this question, in a proposed future *in vivo* study, endogenous FSH and LH secretion would be inhibited in the long-term (by means of a GnRH antagonist or agonist) in control and prenatally androgenised ewes. Exogenous LH would then be administered to controls in a manner that replicates the peripheral LH concentrations and episodic profiles seen in prenatally TP-treated ewes (Sarma *et al.*, 2005), while TP-treated ewes would receive peripheral concentrations in a manner usually seen in controls. The resultant ovarian morphology and

histology, follicular fluid concentrations of oestradiol and progesterone in DF and SF, the follicular expression profiles of *LHR* and *FSHR*, in addition to other genes found to be abnormally regulated, such as *HSD3B1* would then be investigated.

Prenatal androgenisation by DHT reduced granulosa cell steroid production within the DF and SF *in vitro* and that this is most likely an inherent decrease in granulosa cell steroid production. In the future, granulosa cell gene expression (specifically, *LHR*, *FSHR*, *HSD3B1* and *CYP19A1*) in DHT-treated ewes should be investigated to determine if these genes are programmed by the androgenic action of the androgen. As a result of decreased granulosa cell steroid production, we would expect reduced transcript levels of *LHR*, *FSHR*, *HSD3B1* and *CYP19A1*. Results from this study would enable the underlying molecular mechanisms leading to reduced steroid production in DHT-treated ewes and excessive follicle growth in TP-treated ewes to be further elucidated.

This study determined that prenatal androgenisation by TP resulted in premature antral follicle differentiation as seen by the presence of aromatase within antral follicles less than 1 mm in diameter (**Chapter 4**). Premature induction of aromatase in TP-treated ewes implies that these follicles are abnormally differentiated given that aromatase is not normally present until follicles reach a diameter of 3 mm in sheep (Logan *et al.*, 2002). In sheep, FSH is responsible for the induction of aromatase expression in antral follicles (Richards & Hedin, 1988) and small antral follicles less than 1 mm in diameter are not FSH dependent; however they are FSH-responsive (Campbell *et al.*, 2003). Thus, it appears that an FSH-independent mechanism exists resulting in premature follicle differentiation. The most likely candidates that can induce early aromatase induction are various growth factors such as insulin and IGF-1 (Costrici *et al.*, 1994; Campbell *et al.*, 1995; Campbell *et al.*, 2003; Evans, 2003a). Additionally, androgenisation could lead to follicles attaining FSH and LH dependency at an earlier stage of follicle development. To address this hypothesis, a study using immunocytochemistry should be initiated to determine whether FSHR and LHR are present within granulosa cells of small (<1 mm) antral follicles of TP-treated ewes and compared to controls. Within our study, we did try to localise LHR within TP-treated ewes, using an antibody raised against the human receptor (data not shown). However, as is sometimes the case with immunocytochemistry, this antibody did not appear to recognise the ovine ovarian receptor. The use of another antibody might be more successful. An alternative to the use of immunocytochemistry to identify the presence and location of protein would be, *in situ* hybridisation to determine if the expression if *LHR* mRNA is present in granulosa cells. This would determine if

prenatal androgenisation leads to premature follicle differentiation and a possible dependency on LH for follicle growth. Premature follicle differentiation in prenatally TP-treated ewes appears to also apply to the subordinate follicle thus abolishing follicle selection to a certain extent. This presumptive loss in follicle hierarchy in TP-treated animals was also seen during *in vitro* granulosa cell culture, where steroid production from SF was more similar to production from DF in TP animals, when compared to controls.

The reduction in the number of antral follicles when TP-ewes are 22 months of age seen in Chapter 4, together with enhanced follicular depletion (Smith *et al.*, 2008) and increased follicle recruitment from the primordial follicle pool (Steckler *et al.*, 2005; Smith *et al.*, 2008) observed in previous studies in foetal sheep provide considerable evidence that the young prenatally TP-treated ewe is a suitable animal model to study ovarian aging in women (Broekmans *et al.*, 2009). In addition, ovarian abnormalities seen such as a high incidence of cystic degeneration and subluteal function of *corpora lutea*, all characteristics of the ageing ovary in women, are also seen in TP ewes (Unsworth *et al.*, 2005). As the availability of human ovarian tissue is at a premium, this model provides an excellent source of tissue for research purposes. Thus, the prenatally androgenised ewe could be used to investigate further abnormalities (see above) that occur in both premature ovarian aging and timely ovarian aging in women, with the potential to develop novel therapeutic approaches to slow or rescue ovarian function.

Finally, the prenatally TP-treated ewe is seen as a model for PCOS given the similarities between the two (hypersecretion of LH, multifollicular ovaries, hyperinsulinemia) as previously described (Dunaif *et al.*, 1989; Recabarren *et al.*, 2005; Franks *et al.*, 2006; Forsdike *et al.*, 2007; Franks *et al.*, 2008). In terms of antral follicle development, PCOS is characterised by arrested follicle development at the 4-8mm stage (Franks, 1995; Franks *et al.*, 2000; Chang, 2007). However, the TP-treated model, follicles of over 10mm in diameter are regularly observed (our studies and Steckler et al, 2007). Therefore, in the TP-treated ewe, follicles are able to continue to grow past the FSH dependent stage and continue to the LH dependent point of follicle development. Antral follicles within the androgenised ewe may be able to continue to grow due to the presence of an as yet unidentified growth factor of hormone in higher concentrations at a specific stage of development, such as IGF-1, that is not present in PCOS women, or are programmed to respond to the same hormonal milieu (as that seen in PCOS women) by continuing to grow and failing to undergo atresia. Therefore, the prenatally androgenised sheep as an ovarian model for PCOS is a suitable model to study follicle recruitment; however is not an

appropriate animal model to study the later stages of follicle growth (selection and dominance).

Limitations of the Study

There were some limitations of the present study. Firstly, in spite of our best efforts to correctly identify DF and SF from each ewe we were not able to irrefutably determine that those follicles classified into the two groups are correct. In view of this, further studies should utilise transrectal ultrasonography, either with synchronisation of reproductive cycles by prostaglandin F2α in controls and TP animals able to ovulate or when animals are outwith the breeding season, and only follicle waves are monitored, to ensure that both DF and SF are correctly identified. This is of even more importance for granulosa cell culture, where granulosa cells should be isolated from follicles at similar stages of differentiation and within a follicular wave (Jimenez-Krassel & Ireland, 2002). Additionally, post mortem follicle collection (and classification) as was used in this study does not allow correlation of enzyme expression patterns within antral follicles to the circulating endocrine milieu. In the future blood samples should be taken every day for a week prior to euthanasia in order to determine the hormonal milieu before ovary/follicle collection.

Another limitation with our study was that immunofluorescence was used and fluorescent labelling fades, usually after a period of up to eight weeks. Thus, optimum staining is lost beyond this point, and it is sometimes not possible to re-examine microscopic slides several months after initial labelling. Therefore, a detection chemistry other than fluorescence, such as that of the avidin-biotin-peroxidase complex, should be utilised to enable detection and localisation of specific proteins. Additionally, we did not have the option of quantifying CYP19A1 and HSD3B1 enzyme levels in follicle cell layers, as we had to use frozen sections for immunocytochemistry. Frozen sections were used as we were not able detect specific immunoreactivity for these steroidogenic enzymes (using the antibodies previously described) in Bouin's fixed, wax embedded tissue, even when antigen retrieval was performed (data not shown). Using frozen sections it is difficult to determine individual follicular cells accurately within antral follicles and thus it was not possible to determine the proportion of cells immunoreactive for a specific protein (e.g. HSD3B1). Wax embedded tissue would allow determination of granulosa and theca cells and thus, would enable quantification (expressed as a proportion of cells stained) of protein in antral follicles.

Conclusion

The data presented in this thesis have demonstrated for the first time the varied effects of prenatal androgenisation by either TP or DHT on the programming of antral follicle

function within the sheep. This study has shown *in vivo,* that prenatal androgenisation through the oestrogenic action of testosterone increases steroidogenesis and enhances growth of large antral follicles. Excess testosterone *in utero* also results in premature antral follicle differentiation, suggestive of an earlier dependence on LH. In addition, for the first time *in vitro*, we have shown that the androgenic action of testosterone reduces granulosa cell oestradiol production. A major task now is to determine the programming mechanisms *in utero* by excess testosterone which leads to subsequent abnormal antral follicle and specifically granulosa cell function. Results from future studies will provide greater understanding into the long-term effects of prenatal androgenisation, such as the programming of granulosa cell genes, and into the mechanism(s) that result in abnormal antral follicle function.

References

Abbott DH, Foong SC, Barnett DK & Dumesic DA. (2004). Nonhuman primates contribute unique understanding to anovulatory in fertility in women. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources* **45,** 116-131.

Abbott DH, Barnett DK, Bruns CM & Dumesic DA. (2005). Androgen excess fetal programming of female Reproduction: a developmental aetiology for polycystic ovary syndrome? *Human Reproduction Update* **11,** 357-374.

Abbott DH, Zhou R, Bird IM, Dumesic DA & Conley AJ. (2008). Fetal programming of adrenal androgen excess: lessons from a nonhuman primate model of polycystic ovary syndrome. *Endocrine Development* **13,** 145-158.

Abdennebi L, Monget P, Pisselet C, Remy JJ, Salesse R & Monniaux D. (1999). Comparative expression of luteinizing hormone and follicle-stimulating hormone receptors in ovarian follicles from high and low prolific sheep breeds. *Biology of Reproduction* **60,** 845-854.

Alak BM, Coskun S, Friedman CI, Kennard EA, Kim MH & Seifer DB. (1998). Activin A stimulates meiotic maturation of human oocytes and modulates granulosa cell steroidogenesis. *Fertility and Sterility* **70,** 1126-1130.

Amsterdam A, Keren-Tal I, Aharoni D, Dantes A, Land-Bracha A, Rimon E, Sasson R & Hirsh L. (2003). Steroidogenesis and apoptosis in the mammalian ovary. *Steroids* **68,** 861-867.

Arakane F, Kallen CB, Watari H, Foster JA, Sepuri NB, Pain D, Stayrook SE, Lewis M, Gerton GL & Strauss JF, 3rd. (1998). The mechanism of action of steroidogenic acute regulatory protein (StAR). StAR acts on the outside of mitochondria to stimulate steroidogenesis. *The Journal of Biological Chemistry* **273,** 16339-16345.

Armstrong DG, Hogg CO, Campbell BK & Webb R. (1996). Insulin-like growth factor (IGF)-binding protein production by primary cultures of ovine granulosa and theca cells. The effects of IGF-I, gonadotropin, and follicle size. *Biology of Reproduction* **55,** 1163-1171.

Armstrong DG, Gong JG, Gardner JO, Baxter G, Hogg CO & Webb R. (2002). Steroidogenesis in bovine granulosa cells: the effect of short-term changes in dietary intake. *Reproduction (Cambridge, England)* **123,** 371-378.

Auletta FJ & Flint AP. (1988). Mechanisms controlling corpus luteum function in sheep, cows, nonhuman primates, and women especially in relation to the time of luteolysis. *Endocrine Reviews* **9,** 88-105.

Austin EJ, Mihm M, Evans AC, Knight PG, Ireland JL, Ireland JJ & Roche JF. (2001). Alterations in intrafollicular regulatory factors and apoptosis during selection of follicles in the first follicular wave of the bovine estrous cycle. *Biology of Reproduction* **64,** 839-848.

Bacharova R. (1985). Gene expression during oogenesis and oocyte development in mammals. In *Developmental Biology: A Comprehensive Synthesis*, ed. Browder LW, pp. 453-524. Plenum, New York.

Badinga L, Driancourt MA, Savio JD, Wolfenson D, Drost M, De La Sota RL & Thatcher WW. (1992). Endocrine and ovarian responses associated with the first-wave dominant follicle in cattle. *Biology of Reproduction* **47,** 871-883.

Baird DT, Swanston I & Scaramuzzi RJ. (1976). Pulsatile release of LH and secretion of ovarian steroids in sheep during the luteal phase of the estrous cycle. *Endocrinology* **98,** 1490-1496.

Baird DT. (1978). Pulsatile secretion of LH and ovarian estradiol during the follicular phase of the sheep estrous cycle. *Biology of Reproduction* **18,** 359-364.

Baird DT, Campbell BK, Mann GE & McNeilly AS. (1991). Inhibin and oestradiol in the control of FSH secretion in the sheep. *Journal of Reproduction and Fertility Supplement* **43,** 125-138.

Baker TG. (1963). A Quantitative and Cytological Study of Germ Cells in Human Ovaries. *Proceedings of the Royal Society of London Series B, Containing papers of a Biological character* **158,** 417-433.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE & Youngquist RS. (1997b). Expression of messenger ribonucleic acid (mRNA) encoding 3β-hydroxysteroid dehydrogenase Δ^4,Δ^5 isomerase (3β-HSD) during recruitment and selection of bovine ovarian follicles: Identification of dominant follicles by expression of 3β-HSD mRNA within the granulosa cell layer. *Biology of Reproduction* **56,** 1466-1473.

Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE & Youngquist RS. (1997). Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450 side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biology of Reproduction* **56,** 1158-1168.

Bao BN, Calder MD, Xie SC, Smith MF, Salfen BE, Youngquist RS & Garverick HA. (1998a). Expression of steroidogenic acute regulatory protein messenger ribonucleic acid is limited to theca of healthy bovine follicles collected during recruitment, selection, and dominance of follicles of the first follicular wave. *Biology of Reproduction* **59,** 953-959.

Bao B & Garverick HA. (1998b). Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: A review. *Journal of Animal Science* **76,** 1903-1921.

Bao B, Kumar N, Karp RM, Garverick HA & Sundaram K. (2000). Estrogen receptor-β expression in relation to the expression of luteinizing hormone receptor and cytochrome P450 enzymes in rat ovarian follicles. *Biology of Reproduction* **63,** 1747-1755.

Barker DJ. (1997). Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition (Burbank, Los Angeles County, Calif* **13,** 807-813.

Barrell GK, Moenter SM, Caraty A & Karsch FJ. (1992). Seasonal-Changes of Gonadotropin-Releasing-Hormone Secretion in the Ewe. *Biology of Reproduction* **46,** 1130-1135.

Bartlewski PM, Beard AP, Cook SJ, Chandolia RK, Honaramooz A & Rawlings NC. (1999). Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. *Journal of Reproduction and Fertility* **115,** 111-124.

Bartlewski PM, Vanderpol J, Beard AP, Cook SJ & Rawlings NC. (2000). Ovarian antral follicular dynamics and their associations with peripheral concentrations of gonadotropins and ovarian steroids in anoestrous Finnish Landrace ewes. *Animal Reproduction Science* **58,** 273-291.

Bazer FW, Spencer TE & Ott TL. (1997). Interferon tau: a novel pregnancy recognition signal. *American Journal of Reproductive Immunology* **37,** 412-420.

Beg MA, Meira C, Bergfelt DR & Ginther OJ. (2003). Role of oestradiol in growth of follicles and follicle deviation in heifers. *Reproduction* **125,** 847-854.

Bellingham M, Fowler PA, Amezaga MR, Rhind SM, Cotinot C, Mandon-Pepin B, Sharpe RM & Evans NP. (2009). Exposure to a complex cocktail of environmental endocrine-disrupting compounds disturbs the kisspeptin/GPR54 system in ovine hypothalamus and pituitary gland. *Environmental Health Perspectives* **117,** 1556-1562.

Berisha B & Schams D. (2005). Ovarian function in ruminants. *Domestic Animal Endocrinology* **29,** 305-317.

Bindon BM, Blanc MR, Pelletier J, Terqui M & Thimonier J. (1979). Periovulatory gonadotrophin and ovarian steroid patterns in sheep of breeds with differing fecundity. *Journal of Reproduction and Fertility* **55,** 15-25.

Birch RA, Padmanabhan V, Foster DL, Unsworth WP & Robinson JE. (2003). Prenatal programming of reproductive neuroendocrine function: fetal androgen exposure produces progressive disruption of reproductive cycles in sheep. *Endocrinology* **144,** 1426-1434.

Bister JL & Paquay R. (1983). Fluctuations in the plasma levels of the follicle-stimulating hormone during estrous cycle, anestrus, gestation and lactation in the ewe: Evidence for an endogenous rhythm of FSH release. *Theriogenology* **19,** 565-582.

Bodensteiner KJ, Wiltbank MC, Bergfelt DR & Ginther OJ. (1996). Alterations in follicular estradiol and gonadotropin receptors during development of bovine antral follicles. *Theriogenology* **45,** 499-512.

Bodensteiner KJ, Clay CM, Moeller CL & Sawyer HR. (1999). Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biology of Reproduction* **60,** 381-386.

Borwick SC, Rhind SM & McMillan SR. (1994). Ovarian steroidogenesis and development in foetal Scottish Blackface ewes, undernourished in utero from conception. *Journal of Reproduction and Fertility* **Abst. Series 14,** 14.

Borwick SC, Rhind SM & McMillan SR. (1995). Effects of undernutrition from the time of mating and ovarian development in foetal sheep at 62d gestation. *Journal of Reproduction and Fertility* **Abst. Series 15,** 52.

Bove SE, Petroff MG, Nishibori M & Pate JL. (2000). Macrophage migration inhibitory factor in the bovine corpus luteum: characterization of steady-state messenger ribonucleic acid and immunohistochemical localization. *Biology of Reproduction* **62,** 879-885.

Braw-Tal R. (2002). The initiation of follicle growth: the oocyte or the somatic cells? *Molecular and Cellular Endocrinology* **187,** 11-18.

Braw-Tal R, McNatty KP, Smith P, Heath DA, Hudson NL, Phillips DJ, McLeod BJ & Davis GH. (1993). Ovaries of Ewes Homozygous for the X-Linked Inverdale Gene (*FecXI*) Are Devoid of Secondary and Tertiary Follicles but Contain Many Abnormal Structures. *Biology of Reproduction* **49,** 895-907.

Bridges PJ & Fortune JE. (2003). Characteristics of developing prolonged dominant follicles in cattle. *Domestic Animal Endocrinology* **25,** 199-214.

Britt KL, Drummond AE, Dyson M, Wreford NG, Jones ME, Simpson ER & Findlay JK. (2001). The ovarian phenotype of the aromatase knockout (ArKO) mouse. *The Journal of Steroid Biochemistry and Molecular Biology* **79,** 181-185.

Britt KL, Saunders PK, McPherson SJ, Misso ML, Simpson ER & Findlay JK. (2004). Estrogen actions on follicle formation and early follicle development. *Biology of Reproduction* **71,** 1712-1723.

Broekmans FJ, Knauff EA, te Velde ER, Macklon NS & Fauser BC. (2007). Female reproductive ageing: current knowledge and future trends. *Trends in Endocrinology and Metabolism: TEM* **18,** 58-65.

Broekmans FJ, Soules MR & Fauser BC. (2009). Ovarian aging: mechanisms and Clinical consequences. *Endocrine Reviews* **30,** 465-493.

Brooks AN, McNeilly AS & Thomas GB. (1995). Role of GnRH in the ontogeny and regulation of the fetal hypothalamo-pituitary-gonadal axis in sheep. *Journal of Reproduction and Fertility* **49,** 163-175.

Brosens JJ, Tullet J, Varshochi R & Lam EW. (2004). Steroid receptor action. *Best Practice Research in Clinical Obstetrics Gynaecology* **18,** 265-283.

Brown MS & Goldstein JL. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of membrane-bound transcription factor. *Cell* **89,** 331-340.

Bustin SA. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25,** 169-193.

Byskov AG. (1978). Follicular Atresia. In *The Vertebrate Ovary*, ed. Jones R, pp. 533-562. Plenum, New York.

Byskov AG. (1979). In *Ovarian Follicular Development and Function*, ed. Midgley AR & Sadler WA, pp. 41-57. Raven Press, New York.

Cahill LP, Mariana JC & Mauleon P. (1979). Total follicle populations in ewes with high and low ovulation rates. *Journal of Reproduction and Fertility* **55,** 27-36.

Cahill LP & Mauleon P. (1980). Influences of season, cycle and breed on follicular growth rates in sheep. *Journal of Reproduction and Fertility* **58,** 321-328.

Cahill LP & Mauleon P. (1981). A study of the population of primordial and small follicles in the sheep. *Journal of Reproduction and Fertility* **61,** 201-206.

Caldani M, Batailler M, Thiery JC & Dubois MP. (1988). LHRH-immunoreactive structures in the sheep brain. *Histochemistry* **89,** 129-139.

Caldani M, Antoine M, Batailler M & Duittoz A. (1995). Ontogeny of GnRH systems. *Journal of Reproduction and Fertility Supplement* **49,** 147-162.

Calder MD, Manikkam M, Salfen BE, Youngquist RS, Lubahn DB, Lamberson WR & Garverick HA. (2001). Dominant bovine ovarian follicular cysts express increased levels of messenger RNAs for luteinizing hormone receptor and 3 beta-hydroxysteroid dehydrogenase delta(4),delta(5) isomerase compared to normal dominant follicles. *Biology of Reproduction* **65,** 471-476.

Campbell BK, McNeilly AS, Picton HM & Baird DT. (1990). The effect of a potent gonadotrophin-releasing hormone antagonist on ovarian secretion of oestradiol, inhibin and androstenedione and the concentration of LH and FSH during the follicular phase of the sheep oestrous cycle. *The Journal of Endocrinology* **126,** 377-384.

Campbell BK, McNeilly AS, Mann GE & Baird DT. (1991a). The effect of stage of estrous cycle and follicular maturation on ovarian inhibin production in sheep. *Biology of Reproduction* **44,** 483-490.

Campbell BK, Scaramuzzi RJ, Evans G & Downing JA. (1991b). Increased ovulation rate in androstenedione-immune ewes is not due to elevated plasma concentrations of FSH. *Journal of Reproduction and Fertility* **91,** 655-666.

Campbell BK, Scaramuzzi RJ & Webb R. (1995a). Control of Antral Follicle Development and Selection in Sheep and Cattle. *Journal of Reproduction and Fertility* **Suppl. 49,** 335-350.

Campbell BK & Webb R. (1995b). Evidence that inhibin has autocrine and paracrine actions in controlling ovarian function in sheep. *Journal of Reproduction and Fertility* **Abstr. Ser. 15,** 140.

Campbell BK, Scaramuzzi RJ & Webb R. (1996). Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *Journal of Reproduction and Fertility* **106,** 7-16.

Campbell BK, Dobson H & Scaramuzzi RJ. (1998). Ovarian function in ewes made hypogonadal with GnRH antagonist and stimulated with FSH in the presence or absence of low amplitude LH pulses. *The Journal of Endocrinology* **156,** 213-222.

Campbell BK, Dobson H, Baird DT & Scaramuzzi RJ. (1999a). Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep. *Journal of Reproduction and Fertility* **117,** 355-367.

Campbell BK & Baird DT. (2001). Inhibin A is a follicle stimulating hormone-responsive marker of granulosa cell differentiation, which has both autocrine and paracrine actions in sheep. *Journal of Endocrinology* **169,** 333-345.

Campbell BK, Souza C, Gong J, Webb R, Kendall N, Marsters P, Robinson G, Mitchell A, Telfer EE & Baird DT. (2002). Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reproduction (Cambridge, England)*, 429-443.

Campbell BK, Souza C, Gong J, Webb R, Kendall N, Marsters P, Robinson G, Mitchell A, Telfer EE & Baird DT. (2003). Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reproduction (Cambridge, England) Supplement* **61,** 429-443.

Campbell BK, Souza CJ, Skinner AJ, Webb R & Baird DT. (2006). Enhanced response of granulosa and theca cells from sheep carriers of the FecB mutation in vitro to gonadotropins and bone morphogenic protein-2, -4, and -6. *Endocrinology* **147,** 1608-1620.

Carabatsos MJ, Elvin J, Matzuk MM & Albertini DF. (1998). Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Developmental Biology* **204,** 373-384.

Caraty A, Delaleu B, Chesneau D & Fabre-Nys C. (2002). Sequential role of E2 and GnRH for the expression of estrous behavior in ewes. *Endocrinology* **143,** 139-145.

Cardenas H, Burke KA, Bigsby RM, Pope WF & Nephew KP. (2001). Estrogen receptor beta in the sheep ovary during the estrous cycle and early pregnancy. *Biology of Reproduction* **65,** 128-134.

Carroll RS, Corrigan AZ, Gharib SD, Vale W & Chin WW. (1989). Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Molecular Endocrinology (Baltimore, Md* **3,** 1969-1976.

Catt KJ. (1999). Gonadotropin releasing hormone and the GnRH receptor. In *Molecular Biology in Reproductive Medicine*, First edn, ed. Fauser BCJM, Rutherford AJ, Strauss JF & Van Steirteghem A. Parthenon Publishing Group, Carnforth, UK.

Catteau-Jonard S, Jamin SP, Leclerc A, Gonzales J, Dewailly D & di Clemente N. (2008). Anti-Mullerian hormone, its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism* **93,** 4456-4461.

Chaffkin LM, Luciano AA & Peluso JJ. (1993). The role of progesterone in regulating human granulosa cell proliferation and differentiation in vitro. *The Journal of Clinical Endocrinology and Metabolism* **76,** 696-700.

Chang H, Brown CW & Matzuk MM. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocrine Reviews* **23,** 787-823.

Chang RJ. (2007). The reproductive phenotype in polycystic ovary syndrome. *Nature Clinical Practice* **3,** 688-695.

Choi D, Hwang S, Lee E, Yoon S, Yoon BK & Bae D. (2004). Expression of mitochondria-dependent apoptosis genes (p53, Bax, and Bcl-2) in rat granulosa cells during follicular development. *Journal of the Society for Gynecologic Investigation* **11,** 311-317.

Christman GM, Randolph JF, Jr., Peegel H & Menon KM. (1991). Differential responsiveness of luteinized human granulosa cells to gonadotropins and insulin-like growth factor I for induction of aromatase activity. *Fertility and Sterility* **55,** 1099-1105.

Chun SY, Eisenhauer KM, Minami S, Billig H, Perlas E & Hsueh AJW. (1996). Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. *Endocrinology* **137,** 1447-1456.

Clark DA & Coker R. (1998). Transforming growth factor-beta (TGF-beta). *The International Journal of Biochemistry & Cell biology* **30,** 293-298.

Clark DE, Tisdall DJ, Fidler AE & McNatty KP. (1996). Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. *Journal of Reproduction and Fertility* **106,** 329-335.

Clarke IJ, Scaramuzzi RJ & Short RV. (1976). Effects of testosterone implants in pregnant ewes on their female offspring. *Journal of Embryology and Experimental Morphology* **36,** 87-99.

Clarke IJ, Scaramuzzi RJ & Short RV. (1977). Ovulation in prenatally androgenized ewes. *The Journal of Endocrinology* **73,** 385-389.

Clarke IJ & Cummins JT. (1982). The Temporal Relationship between Gonadotropin-Releasing Hormone (Gnrh) and Luteinizing-Hormone (Lh) Secretion in Ovariectomized Ewes. *Endocrinology* **111,** 1737-1739.

Clarke IJ & Cummins JT. (1984). Direct pituitary effects of estrogen and progesterone on gonadotropin secretion in the ovariectomized ewe. *Neuroendocrinology* **39,** 267-274.

Clarke IJ, Thomas GB, Yao B & Cummins JT. (1987). GnRH secretion throughout the ovine estrous cycle. *Neuroendocrinology* **46,** 82-88.

Cleal JK, Poore KR, Newman JP, Noakes DE, Hanson MA & Green LR. (2007). The effect of maternal undernutrition in early gestation on gestation length and fetal and postnatal growth in sheep. *Pediatric research* **62,** 422-427.

Cohen GM. (1997). Caspases: the executioners of apoptosis. *The Biochemical Journal* **326 (Pt 1),** 1-16.

Conley AJ, Kaminski MA, Dubowsky SA, Jablonka-Shariff A, Redmer DA & Reynolds LP. (1995a). Immunohistochemical localization of 3 beta-hydroxysteroid dehydrogenase and P450 17 alpha-hydroxylase during follicular and luteal development in pigs, sheep, and cows. *Biology of Reproduction* **52,** 1081-1094.

Cory S & Adams JM. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nature Reviews* **2,** 647-656.

Costrici N, Lunenfeld B, Pariente C, Dor J, Rabinovici J, Kanety H & Karasik A. (1994). Induction of aromatase in human granulosa cells by both follicle stimulating hormone and insulin-like growth factor-I involves tyrosine phosphorylation. *Gynecology and Endocrinology* **8,** 183-189.

Crisp TM. (1992). Organization of the ovarian follicle and events in its biology: oogenesis, ovulation or atresia. *Mutation Research* **296,** 89-106.

D'Haeseleer M, Cocquyt G, Van Cruchten S, Simoens P & Van den Broeck W. (2006). Cell-specific localisation of apoptosis in the bovine ovary at different stages of the oestrous cycle. *Theriogenology* **65,** 757-772.

Davis GH, McEwan JC, Fennessy PF, Dodds KG & Farquhar PA. (1991). Evidence for the presence of a major gene influencing ovulation rate on the X chromosome of sheep. *Biology of Reproduction* **44,** 620-624.

Davis GH, McEwan JC, Fennessy PF, Dodds KG, McNatty KP & O WS. (1992). InFertility Due to Bilateral Ovarian Hypoplasia in Sheep Homozygous (FecXI FecXI) for the Inverdale Prolificacy Gene Located on the X Chromosome. *Biology of Reproduction* **46,** 636-640.

Deaver DR & Dailey RA. (1983). Effects of dopamine and serotonin on concentrations of luteinizing hormone and estradiol-17 beta in plasma of cycling ewes. *Biology of Reproduction* **28,** 870-877.

DeHaan KC, Berger LL, Kesler DJ, McKeith FK, Thomas DL & Nash TG. (1987). Effect of prenatal androgenization on lamb performance, carcass composition and reproductive function. *Journal of Animal Science* **65,** 1465-1470.

Denamur R. (1968). Formation and maintenance of corpora lutea in domestic animals. *Journal of Animal Science* **27 Suppl 1,** 163-180.

Denning-Kendall PA & Wathes DC. (1994). Acute effects of prostaglandin F2 alpha, luteinizing hormone, and estradiol on second messenger systems and on the secretion of oxytocin and progesterone from granulosa and early luteal cells of the ewe. *Biology of Reproduction* **50,** 765-773.

Diekman MA, O'Callaghan P, Nett TM & Niswender GD. (1978). Validation of methods and quantification of luteal receptors for LH throughout the oestrous cycle and early pregnancy in ewes. *Biology of Reproduction* **19,** 999-1009.

Dong JW, Albertini DF, Nishimori K, Kumar TR, Lu NF & Matzuk MM. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383,** 531-535.

Driancourt MA & Mariana JC. (1982). Short and long-term effects of X-irradiation on ovarian follicular populations in the ewe. *Reproduction, Nutrition, Development* **22,** 813-823.

Driancourt MA, Cahill LP & Bindon BM. (1985). Ovarian Follicular Populations and Preovulatory Enlargement in Booroola and Control Merino Ewes. *Journal of Reproduction and Fertility* **73,** 93-107.

Driancourt MA, Fry RC, Clarke IJ & Cahill LP. (1987). Follicular Growth and Regression During the 8 Days after Hypophysectomy in Sheep. *Journal of Reproduction and Fertility* **79,** 635-641.

Driancourt MA. (1987). Ovarian Features Contributing to the Variability of PMSG-Induced Ovulation Rate in Sheep. *Journal of Reproduction and Fertility* **80,** 207-212.

Draincourt MA, Fair T & Reynaud K. (1998). Oocytary apoptosis. *Contraception Fertilite Sexualite* **26,** 522-527.

Driancourt MA. (2001). Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of Reproduction. *Theriogenology* **55,** 1211-1239.

Drummond AE, Dyson M, Mercer JE & Findlay JK. (1996). Differential responses of post-natal rat ovarian cells to FSH and activin. *Molecular and cellular endocrinology* **122,** 21-32.

Drummond AE, Baillie AJ & Findlay JK. (1999). Ovarian estrogen receptor α and β mRNA expression: impact of development and estrogen. *Molecular and Cellular Endocrinology* **149,** 153-161.

Drummond AE & Findlay JK. (1999b). The role of estrogen in folliculogenesis. *Molecular and Cellular Endocrinology* **151,** 57-64.

Drummond AE, Britt KL, Dyson M, Jones ME, Kerr JB, O'Donnell L, Simpson ER & Findlay JK. (2002). Ovarian steroid receptors and their role in ovarian function. *Molecular and Cellular Endocrinology* **191,** 27-33.

Drummond AE. (2006). The role of steroids in follicular growth. *Reproductive Biology and Endocrinology* **4,** 16.

Drummond AE & Fuller PJ. (2010). The importance of ERbeta signalling in the ovary. *The Journal of Endocrinology* **205,** 15-23.

Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS & Blomme EA. (2003). Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *The Journal of Pathology* **199,** 221-228.

Dufau ML. (1998). The luteinizing hormone receptor. *Annual Review of Physiology* **60,** 461-496.

Dufour J, Cahill LP & Mauleon P. (1979). Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. *Journal of Reproduction and Fertility* **57,** 301-309.

Duggavathi R, Bartlewski PM, Barrett DM, Gratton C, Bagu ET & Rawlings NC. (2004). Patterns of antral follicular wave dynamics and accompanying endocrine changes in cyclic and seasonally anestrous ewes treated with exogenous ovine follicle-stimulating hormone during the inter-wave interval. *Biology of Reproduction* **70,** 821-827.

Duggavathi R, Bartlewski PM, Barrett DM & Rawlings NC. (2005). The temporal relationship between patterns of LH and FSH secretion, and development of ovulatory-sized follicles during the mid- to late-luteal phase of sheep. *Theriogenology* **64,** 393-407.

Duggavathi R, Janardhan K, Singh J, Singh B, Barrett DM, Davies KL, Bagu ET & Rawlings NC. (2006). Patterns of expression of steroidogenic enzymes during the first wave of the ovine estrous cycle as compared to the preovulatory follicle. *Animal Reproduction Science* **91,** 345-352.

Dumesic DA, Abbott DH, Eisner JR & Goy RW. (1997). Prenatal exposure of female rhesus monkeys to testosterone propionate increases serum luteinizing hormone levels in adulthood. *Fertility and Sterility* **67,** 155-163.

Dumesic DA, Schramm RD, Peterson E, Paprocki AM, Zhou R & Abbott DH. (2002). Impaired developmental competence of oocytes in adult prenatally androgenized female rhesus monkeys undergoing gonadotropin stimulation for in vitro fertilization. *The Journal of Clinical Endocrinology and Metabolism* **87,** 1111-1119.

Dumesic DA, Abbott DH & Padmanabhan V. (2007). Polycystic ovary syndrome and its developmental origins. *Reviews: Endocrinology and Metabolic Disorders.*

Dumesic DA, Patankar MS, Barnett DK, Lesnick TG, Hutcherson BA & Abbott DH. (2009). Early prenatal androgenization results in diminished ovarian reserve in adult female rhesus monkeys. *Human Reproduction (Oxford, England)* **24,** 3188-3195.

Dunaif A, Segal KR, Futterweit W & Dobrjansky A. (1989). Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes* **38,** 1165-1174.

Edwards RG, Fowler RE, Gore-Langton RE, Gosden RG, Jones EC, Readhead C & Steptoe PC. (1977). Normal and abnormal follicular growth in mouse, rat and human ovaries. *Journal of Reproduction and Fertility* **51,** 237-263.

Eisner JR, Barnett MA, Dumesic DA & Abbott DH. (2002). Ovarian hyperandrogenism in adult female rhesus monkeys exposed to prenatal androgen excess. *Fertility and Sterility* **77,** 167-172.

Elmore S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology* **35,** 495-516.

Elvin JA, Clark AT, Wang P, Wolfman NM & Matzuk MM. (1999a). Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Molecular Endocrinology* **13,** 1035-1048.

Elvin JA, Yan CN, Wang P, Nishimori K & Matzuk MM. (1999b). Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. *Molecular Endocrinology* **13,** 1018-1034.

Endl E & Gerdes J. (2000). The Ki-67 protein: fascinating forms and an unknown function. *Experimental Cell Research* **257,** 231-237.

Eppig JJ. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122,** 829-838.

Erickson GF, Wang C, Casper R, Mattson G & Hofeditz C. (1982). Studies on the Mechanism of LH Receptor Control by FSH. *Molecular and Cellular Endocrinology* **27,** 17-30.

Eriksson JG & Forsen TJ. (2002). Childhood growth and coronary heart disease in later life. *Annals of Medicine* **34,** 157-161.

Evans AC & Fortune JE. (1997). Selection of the dominant follicle in cattle occurs in the absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors. *Endocrinology* **138,** 2963-2971.

Evans AC, Duffy P, Hynes N & Boland MP. (2000). Waves of follicle development during the estrous cycle in sheep. *Theriogenology* **53,** 699-715.

Evans AC & Martin F. (2000). Kinase pathways in dominant and subordinate ovarian follicles during the first wave of follicular development in sheep. *Animal Reproduction Science* **64,** 221-231.

Evans AC. (2003a). Characteristics of ovarian follicle development in domestic animals. *Reproduction in Domestic Animals = Zuchthygiene* **38,** 240-246.

Evans AC. (2003b). Ovarian follicle growth and consequences for Fertility in sheep. *Animal Reproduction Science* **78,** 289-306.

Evans AC, Ireland JL, Winn ME, Lonergan P, Smith GW, Coussens PM & Ireland JJ. (2004). Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle. *Biology of Reproduction* **70,** 1475-1484.

Evans NP, Dahl GE, Glover BH & Karsch FJ. (1994). Central regulation of pulsatile gonadotropin-releasing hormone (GnRH) secretion by estradiol during the period leading up to the preovulatory GnRH surge in the ewe. *Endocrinology* **134,** 1806-1811.

Evans NP, Dahl GE, Caraty A, Padmanabhan V, Thrun LA & Karsch FJ. (1996). How much of the gonadotropin-releasing hormone (GnRH) surge is required for generation of the luteinizing hormone surge in the ewe? Duration of the endogenous GnRH signal. *Endocrinology* **137,** 4730-4737.

Feranil J, Isobe N & Nakao T. (2005). Apoptosis in the antral follicles of swamp buffalo and cattle ovary: TUNEL and caspase-3 histochemistry. *Reproduction in Domestic Animals = Zuchthygiene* **40,** 111-116.

Findlay JK. (1993). An Update on the Roles of Inhibin, Activin, and Follistatin as Local Regulators of Folliculogenesis. *Biology of Reproduction* **48,** 15-23.

Findlay JK & Drummond AE. (1996). Control of follicular growth. In *Symposium on the ovary: regulation, dysfunction and treatment*, pp. 13-21. Elsevier Science B.V., Amsterdam, Florida.

Findlay JK, Drummond AE, Britt KL, Dyson M, Wreford NG, Robertson DM, Groome NP, Jones ME & Simpson ER. (2000). The roles of activins, inhibins and estrogen in early committed follicles. *Molecular and Cellular Endocrinology* **163,** 81-87.

Findlay JK, Drummond AE, Dyson M, Baillie AJ, Robertson DM & Ethier JF. (2001). Production and actions of inhibin and activin during folliculogenesis in the rat. *Molecular and Cellular Endocrinology* **180,** 139-144.

Findlay JK, Drummond AE, Dyson ML, Baillie AJ, Robertson DM & Ethier JF. (2002). Recruitment and development of the follicle; the roles of the transforming growth factor-β superfamily. *Molecular and Cellular Endocrinology* **191,** 35-43.

Flaws JA, Kugu K, Trbovich AM, Desanti A, Tilly KI, Hirshfield AN & Tilly JL. (1995). Interleukin-1β-Converting Enzyme-Related Proteases (IRPs) and Mammalian Cell Death: Dissociation of IRP-Induced Oligonucleosomal Endonuclease Activity from Morphological Apoptosis in Granulosa Cells of the Ovarian Follicle. *Endocrinology* **136,** 5042-5053.

Flaws JA, Hirshfield AN, Hewitt JA, Babus JK & Furth PA. (2001). Effect of *bcl-2* on the primordial follicle endowment in the mouse ovary. *Biology of Reproduction* **64,** 1153-1159.

Flynn JD, Duffy P, Boland MP & Evans AC. (2000). Progestagen synchronisation in the absence of a corpus luteum results in the ovulation of a persistent follicle in cyclic ewe lambs. *Animal Reproduction Science* **62,** 285-296.

Foecking EM, Szabo M, Schwartz NB & Levine JE. (2005). Neuroendocrine consequences of prenatal androgen exposure in the female rat: absence of luteinizing hormone surges, suppression of progesterone receptor gene expression, and acceleration of the gonadotropin-releasing hormone pulse generator. *Biology of Reproduction* **72,** 1475-1483.

Forde N, Mihm M, Canty MJ, Zielak AE, Baker PJ, Park S, Lonergan P, Smith GW, Coussens PM, Ireland JJ & Evans AC. (2008). Differential expression of signal transduction factors in ovarian follicle development: a functional role for betaglycan and FIBP in granulosa cells in cattle *Physiological Genomics* **33,** 193-204.

Forsdike RA, Hardy K, Bull L, Stark J, Webber LJ, Stubbs S, Robinson JE & Franks S. (2007). Disordered follicle development in ovaries of prenatally androgenized ewes. *The Journal of Endocrinology* **192,** 421-428.

Fortune JE & Quirk SM. (1988). Regulation of steroidogenesis in bovine preovulatory follicles. *Journal of Animal Science* **66,** 1-8.

Fortune JE. (1994). Ovarian Follicular Growth and Development in Mammals. *Biology of Reproduction* **50,** 225-232.

Fortune JE, Rivera GM, Evans ACO & Turzillo AM. (2001). Differentiation of dominant versus subordinate follicles in cattle. *Biology of Reproduction* **65,** 648-654.

Fortune JE. (2003). The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal Reproduction Science* **78,** 135-163.

Fortune JE, Rivera GM & Yang MY. (2004). Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. *Animal Reproduction science* **82-83,** 109-126.

Fortune JE & Vincent SE. (1983). Progesterone inhibits the induction of aromatase activity in rat granulosa cells in vitro. *Biology of Reproduction* **28,** 1078-1089.

Foster DL, Ryan KD, Goodman RL, Legan SJ, Karsch FJ & Yellon SM. (1986). Delayed puberty in lambs chronically treated with oestradiol. *Journal of Reproduction and Fertility* **78,** 111-117.

Foster DL. (1994). Puberty in the sheep. In: Knobil E, Neil JD (eds) T*he Physiology of Reproduction*, ed 2. Raven Press, New York, pp 411–451

Foster DL, Padmanabhan V, Wood RI & Robinson JE. (2002). Sexual differentiation of the neuroendocrine control of gonadotrophin secretion: concepts derived from sheep models. *Reproduction (Cambridge, England) Supplement* **59,** 83-99.

Franks S. (1995). Polycystic ovary syndrome. *The New England Journal of Medicine* **333,** 853-861.

Franks S, Gharani N, Waterworth D, Batty S, White D, Williamson R & McCarthy M. (1997). The genetic basis of polycystic ovary syndrome. *Human Reproduction (Oxford, England)* **12,** 2641-2648.

Franks S, Mason H & Willis D. (2000). Follicular dynamics in the polycystic ovary syndrome. *Molecular and Cellular Endocrinology* **163,** 49-52.

Franks S, McCarthy MI & Hardy K. (2006). Development of polycystic ovary syndrome: involvement of genetic and environmental factors. *International Journal of Andrology* **29,** 278-285; discussion 286-290.

Franks S, Stark J & Hardy K. (2008). Follicle dynamics and anovulation in polycystic ovary syndrome. *Human Reproduction Update* **14,** 367-378.

Fry RC & Driancourt MA. (1996). Relationships between follicle-stimulating hormone, follicle growth and ovulation rate in sheep. *Reproduction, Fertility, and Development* **8,** 279-286.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MPE, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH & Ritvos O. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and inFertility in a dosage-sensitive manner. *Nature Genetics* **25,** 279-283.

Gastal EL, Gastal MO, Nogueira GP, Bergfelt DR & Ginther OJ. (2000). Temporal interrelationships among luteolysis, FSH and LH concentrations and follicle deviation in mares. *Theriogenology* **53,** 925-940.

Ginther OJ, Knopf L & Kastelic JP. (1989). Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. *Journal of Reproduction and Fertility* **87,** 223-230.

Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR & Kot K. (1996). Selection of the dominant follicle in cattle. *Biology of Reproduction* **55,** 1187-1194.

Ginther OJ, Kot K, Kulick LJ & Wiltbank MC. (1997). Sampling follicular fluid without altering follicular status in cattle: Oestradiol concentrations early in a follicular wave. *Journal of Reproduction and Fertility* **109,** 181-186.

Ginther OJ, Bergfelt DR, Kulick LJ & Kot K. (1999). Selection of the dominant follicle in cattle: Establishment of follicle deviation in less than 8 hours through depression of FSH concentrations. *Theriogenology* **52,** 1079-1093.

Ginther OJ, Bergfelt DR, Kulick LJ & Kot K. (2000a). Selection of the dominant follicle in cattle: Role of estradiol. *Biology of Reproduction* **63,** 383-389.

Ginther OJ, Bergfelt DR, Kulick LJ & Kot K. (2000b). Selection of the dominant follicle in cattle: Role of two-way functional coupling between follicle-stimulating hormone and the follicles. *Biology of Reproduction* **62,** 920-927.

Glister C, Tannetta DS, Groome NP & Knight PG. (2001). Interactions between follicle-stimulating hormone and growth factors in modulating secretion of steroids and inhibin-related peptides by nonluteinized bovine granulosa cells. *Biology of Reproduction* **65,** 1020-1028.

Glister C, Richards SL & Knight PG. (2005). Bone morphogenetic proteins (BMP) -4, -6, and -7 potently suppress basal and luteinizing hormone-induced androgen production by bovine theca interna cells in primary culture: could ovarian hyperandrogenic dysfunction be caused by a defect in thecal BMP signaling? *Endocrinology* **146,** 1883-1892.

Glister C, Groome NP & Knight PG. (2006). Bovine follicle development is associated with divergent changes in activin-A, inhibin-A and follistatin and the relative abundance of different follistatin isoforms in follicular fluid. *The Journal of Endocrinology* **188,** 215-225.

Goding JR. (1974). The demonstration that PGF2alpha is the uterine luteolysin in the ewe. *Journal of Reproduction and Fertility* **38,** 261-271.

Godsland IF, Walton C, Felton C, Proudler A, Patel A & Wynn V. (1992). Insulin resistance, secretion, and metabolism in users of oral contraceptives. *The Journal of Clinical Endocrinology and Metabolism* **74,** 64-70.

Gong JG, McBride D, Bramley TA & Webb R. (1994). Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on bovine granulosa cell steroidogenesis in vitro. *The Journal of Endocrinology* **143,** 157-164.

Goodman RL. (1993). Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neill JD (eds) *The Physiology of Reproduction, ed 2* **vol 2,** 659–709.

Goodman RL. (1994). Neuroendocrine control of the ovine estrous cycle. *In:The Physiology of Reproduction (Editors: E Knobil and JD Neill), 2nd Edition Raven Press, New York pp 660-93.*

Gore-Langton RE & Dorrington JH. (1981). FSH induction of aromatase in cultured rat granulosa cells measured by a radiometric assay. *Molecular and Cellular Endocrinology* **22,** 135-151.

Gore-Langton RE & Daniel SA. (1990). Follicle-stimulating hormone and estradiol regulate antrum-like reorganization of granulosa cells in rat preantral follicle cultures. *Biology of Reproduction* **43,** 65-72.

Gorski RA. (1986). Sexual differentiation of the brain: a model for drug-induced alterations of the reproductive system. *Environmental Health Perspectives* **70,** 163-175.

Gosden RG & Telfer E. (1987). Numbers of Follicles and Oocytes in Mammalian Ovaries and Their Allometric Relationships. *Journal of Zoology* **211,** 169-175.

Gosden RG, Hunter RHF, Telfer E, Torrance C & Brown N. (1988). Physiological Factors Underlying the Formation of Ovarian Follicular-Fluid. *Journal of Reproduction and Fertility* **82,** 813-825.

Gown AM & Willingham MC. (2002). Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J Histochemistry and Cytochemistry* **50,** 449-454.

Gromoll J & Simoni M. (2005). Genetic complexity of FSH receptor function. *Trends in Endocrinology and Metabolism: TEM* **16,** 368-373.

Gruber CJ, Tschugguel W, Schneeberger C & Huber JC. (2002). Production and actions of estrogens. *The New England Journal of Medicine* **346,** 340-352.

Guthrie HD, Grimes RW, Cooper BS & Hammond JM. (1995). Follicular Atresia in Pigs - Measurement and Physiology. *Journal of Animal Science* **73,** 2834-2844.

Gutierrez CG, Campbell BK & Webb R. (1997). Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. *Biology of Reproduction* **56,** 608-616.

Gwynne JT & Strauss JF. (1982). The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocrine Reviews* **3,** 295-329.

Hafez ESE. (1993a). Folliculogenesis, Egg Maturation, and Ovulation. In *Reproduction in Farm Animals*, Sixth edn, ed. Hafez ESE, pp. 114-143. Lea & Febiger, Philadelphia.

Hall DG, Fogarty NM & Gilmour AR. (1986). Seasonality of ovulation and estrus, and the ram effect in poll dorset ewes. *Theriogenology* **25,** 455-461.

Hartley PS, Bayne RA, Robinson LL, Fulton N & Anderson RA. (2002). Developmental changes in expression of myeloid cell leukemia-1 in human germ cells during oogenesis and early folliculogenesis. *The Journal of Clinical Endocrinology and Metabolism* **87,** 3417-3427.

Hauger RL, Karsch FJ & Foster DL. (1977). A new concept for control of the estrous cycle of the ewe based on the temporal relationships between luteinizing hormone, estradiol and progesterone in peripheral serum and evidence that progesterone inhibits tonic LH secretion. *Endocrinology* **101,** 807-817.

Hay MF & Moor RM. (1975). Functional and structural relationships in the Graafian follicle population of the sheep ovary. *Journal of Reproduction and Fertility* **45,** 583-593.

Hayashi M, McGee EA, Min G, Klein C, Rose UM, van Duin M & Hsueh AJW. (1999). Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology* **140,** 1236-1244.

Herbosa CG, Dahl GE, Evans NP, Pelt J, Wood RI & Foster DL. (1996). Sexual differentiation of the surge mode of gonadotropin secretion: prenatal androgens abolish the gonadotropin-releasing hormone surge in the sheep. *Journal of Neuroendocrinology* **8,** 627-633.

Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L & Richards JS. (2006). Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Molecular Endocrinology (Baltimore, Md* **20,** 1300-1321.

Hillier SG. (1991). Regulatory functions for inhibin and activin in human ovaries. *Journal of Endocrinology* **131,** 171-175.

Hillier SG & Miro F. (1993). Inhibin, activin and follistatin: potential roles in ovarian physiology. *Annals of the New York Academy of Sciences* **687,** 29-38.

Hillier SG. (2001). Gonadotropic control of ovarian follicular growth and development. *Molecular and cellular Endocrinology* **179,** 39-46.

Hinch GN & Roelofs JHW. (1986). Lupin feeding and insulin infusion during the late luteal phase can increase ovulation rate in sheep. In *Proceedings of the Australian Society of Reproductive Biology*, pp. 43.

Hirshfield AN & Midgley AR. (1978). Morphometric analysis of follicular development in the rat. *Biology of Reproduction* **19,** 597-605.

Hirshfield AN. (1984). Stathmokinetic analysis of granulosa cell proliferation in antral follicles of cyclic rats. *Biology of Reproduction* **31,** 52-58.

Hirshfield AN. (1986). Patterns of [^{3}H] Thymidine Incorporation Differ in Immature Rats and Mature, Cycling Rats. *Biology of Reproduction* **34,** 229-235.

Hirshfield AN. (1989a). Granulosa Cell Proliferation in Very Small Follicles of Cycling Rats Studied by Long-Term Continuous Tritiated-Thymidine Infusion. *Biology of Reproduction* **41,** 309-316.

Hirshfield AN. (1989b). Rescue of Atretic Follicles in vitro and in vivo. *Biology of Reproduction* **40,** 181-190.

Hirshfield AN. (1991). Development of Follicles in the Mammalian Ovary. *International Review of Cytology* **124,** 43-101.

Hodgen GD. (1982). The dominant ovarian follicle. *Fertility and Sterility* **38,** 281-300.

Holemans K, Aerts L & Van Assche FA. (1998). Fetal growth and long-term consequences in animal models of growth retardation. *European Journal of Obstetrics, Gynecology, and Reproductive biology* **81,** 149-156.

Hsu SY & Hsueh AJW. (2000). Tissue-specific BCL-2 protein partners in apoptosis: An ovarian paradigm. *Physiological Reviews* **80,** 593-614.

Hsueh AJW, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW & Vale W. (1987). Heterodimers and Homodimers of Inhibin Subunits Have Different Paracrine Action in the Modulation of Luteinizing Hormone-Stimulated Androgen Biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 5082-5086.

Hsueh AJW, Billig H & Tsafriri A. (1994). Ovarian Follicle Atresia - a Hormonally Controlled Apoptotic Process. *Endocrine Reviews* **15,** 707-724.

Huet C, Monget P, Pisselet C & Monniaux D. (1997). Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. *Biology of Reproduction* **56,** 1025-1034.

Hughes IA. (1998). The masculinized female and investigation of abnormal sexual development. *Bailliere's Clinical Endocrinology and Metabolism* **12,** 157-171.

Hughesdon PE. (1982). Morphology and morphogenesis of the Stein-Leventhal ovary and of so-called "hyperthecosis". *Obstetrical & Gynecological survey* **37,** 59-77.

Hulshof SC, Figueiredo JR, Beckers JF, Bevers MM, van der Donk JA & van den Hurk R. (1995). Effects of fetal bovine serum, FSH, and 17β-estradiol on the culture of bovine preantral follicles. *Theriogenology* **44,** 217-226.

Hutchinson LA, Findlay JK, de Vos FL & Robertson DM. (1987). Effects of bovine inhibin, transforming growth factor-beta and bovine Activin-A on granulosa cell differentiation. *Biochemical and Biophysical Research Communications* **146,** 1405-1412.

Igney FH & Krammer PH. (2002). Death and anti-death: tumour resistance to apoptosis. *Nature Reviews* **2,** 277-288.

Ireland JJ & Roche JF. (1983a). Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. *Endocrinology* **112,** 150-156.

Ireland JJ & Roche JF. (1983b). Growth and differentiation of large antral follicles after spontaneous luteolysis in heifers: changes in concentration of hormones in follicular fluid and specific binding of gonadotropins to follicles. *Journal of Animal Science* **57,** 157-167.

Ireland JL, Jimenez-Krassel F, Winn ME, Burns DS & Ireland JJ. (2004). Evidence for autocrine or paracrine roles of alpha2-macroglobulin in regulation of estradiol production by granulosa cells and development of dominant follicles. *Endocrinology* **145,** 2784-2794.

Ireland JL, Scheetz D, Jimenez-Krassel F, Themmen AP, Ward F, Lonergan P, Smith GW, Perez GI, Evans AC & Ireland JJ. (2008). Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biology of Reproduction* **79,** 1219-1225.

Irvine CH & Alexander SL. (1987). A novel technique for measuring hypothalamic and pituitary hormone secretion rates from collection of pituitary venous effluent in the normal horse. *The Journal of Endocrinology* **113,** 183-192.

Irving-Rodgers HF, van Wezel IL, Mussard ML, Kinder JE & Rodgers RJ. (2001). Atresia revisited: two basic patterns of atresia of bovine antral follicles. *Reproduction (Cambridge, England)* **122,** 761-775.

Izawa M, Nguyen PH, Kim HH & Yeh J. (1998). Expression of the apoptosis-related genes, caspase-1, caspase-3, DNA fragmentation factor, and apoptotic protease activating factor-1, in human granulosa cells. *Fertility and Sterility* **70,** 549-552.

Jackson LM, Timmer KM & Foster DL. (2009). Organizational actions of postnatal estradiol in female sheep treated prenatally with testosterone: programming of prepubertal neuroendocrine function and the onset of puberty. *Endocrinology* **150,** 2317-2324.

Jacobs HS. (1969). Use of activated charcoal in the radioimmunoassay of human growth hormone in plasma. *Journal of Clinical Pathology* **22,** 710-717.

Jakimiuk AJ, Weitsman SR, Brzechffa PR & Magoffin DA. (1998). Aromatase mRNA expression in individual follicles from polycystic ovaries. *Molecular Human Reproduction* **4,** 1-8.

Jakimiuk AJ, Weitsman SR, Navab A & Magoffin DA. (2001). Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *The Journal of Clinical Endocrinology and Metabolism* **86,** 1318-1323.

Jansson N, Pettersson J, Haafiz A, Ericsson A, Palmberg I, Tranberg M, Ganapathy V, Powell TL & Jansson T. (2006). Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. *The Journal of Physiology* **576,** 935-946.

Jansson N, Nilsfelt A, Gellerstedt M, Wennergren M, Rossander-Hulthen L, Powell TL & Jansson T. (2008). Maternal hormones linking maternal body mass index and dietary intake to birth weight. *The American Journal of Clinical Nutrition* **87,** 1743-1749.

Jimenez-Krassel F & Ireland JJ. (2002). Development and validation of a short-term, serum-free culture system for bovine granulosa cells: evaluation of the effects of somatotropin and growth hormone-releasing factor on estradiol production. *Journal of Dairy Science* **85,** 68-78.

Jimenez-Krassel F, Winn ME, Burns D, Ireland JL & Ireland JJ. (2003). Evidence for a negative intrafollicular role for inhibin in regulation of estradiol production by granulosa cells. *Endocrinology* **144,** 1876-1886.

Johnson AL, Li Z & Tilly JL. (1993). Expression of *c-myc* and *bcl-2* mRNA during ovarian follicle development. *Biology of Reproduction* **48 (Suppl. 1),** 152.

Johnson AL & Bridgham JT. (2002). Caspase-mediated apoptosis in the vertebrate ovary. *Reproduction (Cambridge, England)* **124,** 19-27.

Jolly PD, Tisdall DJ, Heath DA, Lun S & McNatty KP. (1994). Apoptosis in Bovine Granulosa Cells in Relation to Steroid Synthesis, Cyclic Adenosine-3',5'-Monophosphate Response to Follicle-Stimulating Hormone and Luteinizing Hormone, and Follicular Atresia. *Biology of Reproduction* **51,** 934-944.

Jolly PD, Smith PR, Heath DA, Hudson NL, Lun S, Still LA, Watts CH & McNatty KP. (1997). Morphological evidence of apoptosis and the prevalence of apoptotic versus mitotic cells in the membrana granulosa of ovarian follicles during spontaneous and induced atresia in ewes. *Biology of Reproduction* **56,** 837-846.

Jolly PD, Tisdall DJ, Death G, Heath DA, Lun S, Hudson NL & McNatty KP. (1997b). Granulosa cell apoptosis, aromatase activity, cyclic adenosine 3',5'-monophosphate response to gonadotropins, and follicular fluid steroid levels during spontaneous and induced follicular atresia in ewes. *Biology of Reproduction* **56,** 830-836.

Jost A, Vigier B, Prepin J & Perchellet JP. (1973). Studies on sex differentiation in mammals. *Recent Progress in Hormone Research* **29,** 1-41.

Joyce IM, Clark AT, Pendola FL & Eppig JJ. (2000). Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles. *Biology of Reproduction* **63,** 1669-1675.

Juengel JL, Heath DA, Quirke LD & McNatty KP. (2006). Oestrogen receptor alpha and beta, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep. *Reproduction (Cambridge, England)* **131,** 81-92.

Juengel JL, Sawyer HR, Smith PR, Quirke LD, Heath DA, Lun S, Wakefield SJ & McNatty KP. (2002). Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. *Molecular and Cellular Endocrinology* **191,** 1-10.

Kanzaki M, Hattori M-A, Horiuchi R & Kojima I. (1994). Coordinate actions of FSH and IGF-I on LH receptor expression in rat granulosa cells. *Journal of Endocrinology* **141,** 301-308.

Karsch FJ. (1987). Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. *Annual Review of Physiology* **49,** 365-382.

Kastelic JP, Knopf L & Ginther OJ. (1990). Effect of Day of Prostaglandin $F_{2\alpha}$ Treatment on Selection and Development of the Ovulatory Follicle in Heifers. *Animal Reproduction Science* **23,** 169-180.

Katz M & Carr PJ. (1976). Abnormal luteinizing hormone response patterns to synthetic gonadotrophin releasing hormone in patients with polycystic ovarian syndrome. *The Journal of Endocrinology* **70,** 163-171.

Kaynard AH, Malpaux B, Robinson JE, Wayne NL & Karsch FJ. (1988). Importance of pituitary and neural actions of estradiol in induction of the luteinizing hormone surge in the ewe. *Neuroendocrinology* **48,** 296-303.

Khoury RH, Wang QF, Crowley WF, Jr., Hall JE, Schneyer AL, Toth T, Midgley AR, Jr. & Sluss PM. (1995). Serum follistatin levels in women: evidence against an endocrine function of ovarian follistatin. *The Journal of Clinical Endocrinology and Metabolism* **80,** 1361-1368.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH & Peter ME. (1995). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *The EMBO Journal* **14,** 5579-5588.

Knight PG. (1996). Roles of inhibins, activins, and follistatin in the female reproductive system. *Frontiers in Neuroendocrinology* **17,** 476-509.

Knight PG, Feist SA, Tannetta DS, Bleach EC, Fowler PA, O'Brien M & Groome NP. (1998). Measurement of inhibin-A (alpha beta A dimer) during the oestrous cycle, after manipulation of ovarian activity and during pregnancy in ewes. *Journal of reproduction and fertility* **113,** 159-166.

Knight PG & Glister C. (2001). Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction* **121,** 503-512.

Knopf L, Kastelic JP, Schallenberger E & Ginther OJ. (1989). Ovarian follicular dynamics of heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. *Domestic Animal Endocrinology* **6,** 111-119.

Kojima N, Stumpf TT, Cupp AS, Werth LA, Roberson MS, Wolfe MW, Kittok RJ & Kinder JE. (1992). Exogenous progesterone and progestins as used in estrous synchrony regimens do not mimic the corpus luteum in regulation of luteinizing hormone and 17 beta-estradiol in circulation of cows. *Biology of Reproduction* **47,** 1009-1017.

Kojima AY, Kobayashi S, Acosta TJ, Kudo M, Miyamoto A, Takagi M, Miyazawa K & Sato K. (2002). Effects of LH and PGF(2α) in equine dominant follicles observed by MDS. *Journal of Veterinary Medical Science* **64,** 119-122.

Krajewski S, Bodrug S, Krajewska M, Shabaik A, Gascoyne R, Berean K & Reed JC. (1995). Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. *The American journal of pathology* **146,** 1309-1319.

Krarup T, Pedersen T & Faber M. (1969). Regulation of oocyte growth in the mouse ovary. *Nature* **224,** 187-188.

Kugu K, Ratts VS, Piquette GN, Tilly KI, Tao XJ, Martimbeau S, Aberdeen GW, Krajewski S, Reed JC, Pepe GJ, Albrecht ED & Tilly JL. (1998). Analysis of apoptosis and expression of bcl-2 gene family members in the human and baboon ovary. *Cell Death and Differentiation* **5,** 67-76.

Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson J-A. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 5925-5930.

Langhout DJ, Spicer LJ & Geisert RD. (1991). Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *Journal of Animal Science* **69,** 3321-3334.

Lapolt PS, Oikawa M, Jia XC, Dargan C & Hsueh AJW. (1990). Gonadotropin-Induced up-Regulation and Down-Regulation of Rat Ovarian LH Receptor Message Levels During Follicular Growth, Ovulation and Luteinization. *Endocrinology* **126,** 3277-3279.

Lehman MN, Robinson JE, Karsch FJ & Silverman AJ. (1986). Immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) pathways in the sheep brain during anestrus and the mid-luteal phase of the estrous cycle. *The Journal of Comparative Neurology* **244,** 19-35.

Levine JE & Ramirez VD. (1982). Luteinizing hormone-releasing hormone release during the rat estrous cycle and after ovariectomy, as estimated with push-pull cannulae. *Endocrinology* **111,** 1439-1448.

Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikjian LM & Vale W. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404,** 411-414.

Li R, Phillips DM & Mather JP. (1995). Activin promotes ovarian follicle development in vitro. *Endocrinology* **136,** 849-856.

Lin HY & Moustakas A. (1994). TGF-beta receptors: structure and function. *Cellular and Molecular Biology (Noisy-le-Grand, France)* **40,** 337-349.

Lin D, Sugawara T, Strauss JF, 3rd, Clark BJ, Stocco DM, Saenger P, Rogol A & Miller WL. (1995). Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science (New York, NY* **267,** 1828-1831.

Lin SY, Morrison JR, Phillips DJ & de Kretser DM. (2003). Regulation of ovarian function by the TGF-beta superfamily and follistatin. *Reproduction (Cambridge, England)* **126,** 133-148.

Ling N, Ueno N, Ying SY, Esch F, Shimasaki S, Hotta M, Cuevas P & Guillemin R. (1988). Inhibins and activins. *Vitamins and Hormones* **44,** 1-46.

Liu JY, Aronow BJ, Witte DP, Pope WF & La Barbera AR. (1998). Cyclic and maturation-dependent regulation of follicle-stimulating hormone receptor and luteinizing hormone receptor messenger ribonucleic acid expression in the porcine ovary. *Biology of Reproduction* **58,** 648-658.

Locksley RM, Killeen N & Lenardo MJ. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104,** 487-501.

Logan, KE. (2009). Real-time PCR: current technology and applications p285-310

Logan KA, Juengel JL & McNatty KP. (2002). Onset of steroidogenic enzyme gene expression during ovarian follicular development in sheep. *Biology of Reproduction* **66,** 906-916.

Luck MR, Rodgers RJ & Findlay JK. (1990). Secretion and gene expression of inhibin, oxytocin and steroid hormones during the in vitro differentiation of bovine granulosa cells. *Reproduction, Fertility, and Development* **2,** 11-25.

Lucy MC. (2007). The bovine dominant ovarian follicle. *Journal of Animal Science*. **134**. 344-356

Luderer U, Diaz D, Faustman EM & Kavanagh TJ. (2003). Localization of glutamate cysteine ligase subunit mRNA within the rat ovary and relationship to follicular apoptosis. *Molecular Reproduction and Development* **65,** 254-261.

Lund SA, Murdoch J, Van Kirk EA & Murdoch WJ. (1999). Mitogenic and antioxidant mechanisms of estradiol action in preovulatory ovine follicles: relevance to luteal function. *Biology of Reproduction* **61,** 388-392.

Luther J, Aitken R, Milne J, Matsuzaki M, Reynolds L, Redmer D & Wallace J. (2007). Maternal and fetal growth, body composition, endocrinology, and metabolic status in undernourished adolescent sheep. *Biology of Reproduction* **77,** 343-350.

Maciel GA, Baracat EC, Benda JA, Markham SM, Hensinger K, Chang RJ & Erickson GF. (2004). Stockpiling of transitional and classic primary follicles in ovaries of women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism* **89,** 5321-5327.

Magoffin DA & Weitsman SR. (1994). IGF-1 regulation of LH receptor mRNA expression and LH-stimulated signal transduction in rat ovarian theca-interstitial cells. *Biology of Reproduction* **51,** 766-775.

Malcolm KD, Jackson LM, Bergeon C, Lee TM, Padmanabhan V & Foster DL. (2006). Long-term exposure of female sheep to physiologic concentrations of estradiol: effects on the onset and maintenance of reproductive function, pregnancy, and social development in female offspring. *Biology of Reproduction* **75,** 844-852.

Mani AM, Fenwick MA, Cheng Z, Sharma MK, Singh D & Wathes DC. (2010). IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. *Reproduction (Cambridge, England)* **139,** 139-151.

Manikkam M, Crespi EJ, Doop DD, Herkimer C, Lee JS, Yu S, Brown MB, Foster DL & Padmanabhan V. (2004). Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. *Endocrinology* **145,** 790-798.

Manikkam M, Steckler TL, Welch KB, Inskeep EK & Padmanabhan V. (2006). Fetal programming: prenatal testosterone treatment leads to follicular persistence/luteal defects; partial restoration of ovarian function by cyclic progesterone treatment. *Endocrinology* **147,** 1997-2007.

Manikkam M, Thompson RC, Herkimer C, Welch KB, Flak J, Karsch FJ & Padmanabhan V. (2008). Developmental programming: impact of prenatal testosterone excess on pre- and postnatal gonadotropin regulation in sheep. *Biology of Reproduction* **78,** 648-660.

Manuel Silva J & Price CA. (2000). Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells in vitro. *Biology of Reproduction* **62,** 186-191.

Marion GB, Gier HT & Choudary JB. (1968). Micromorphology of the bovine ovarian follicular system. *Journal of Animal Science* **27,** 451-465.

Masek KS, Wood RI & Foster DL. (1999). Prenatal dihydrotestosterone differentially masculinizes tonic and surge modes of luteinizing hormone secretion in sheep. *Endocrinology* **140,** 3459-3466.

Massague J. (1990). The transforming growth factor-beta family. *Annual Review of Cell Biology* **6,** 597-641.

Matamoros IA, Cox NM & Moore AB. (1991). Effects of exogenous insulin and body condition on metabolic hormones and gonadotropin induced follicular development in pre-pubertal gilts. *Journal of Animal Science* **69,** 2081-2091.

Matikainen T, Perez GI, Zheng TS, Kluzak TR, Rueda BR, Flavell RA & Tilly JL. (2001). Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. *Endocrinology* **142,** 2468-2480.

Matwijiw I & Faiman C. (1989). Control of gonadotropin secretion in the ovine fetus. II. A sex difference in pulsatile luteinizing hormone secretion after castration. *Endocrinology* **124,** 1352-1358.

McClellan MC, Diekman MA, Able JH & Niswender GD. (1975). Luteinizing hormone, progesterone and the morphological development of normal and superovulated corpora lutea in sheep. *Cell and Tissue Research* **164,** 291-307.

McConnell DS, Wang Q, Sluss PM, Bolf N, Khoury RH, Schneyer AL, Midgley AR, Jr., Reame NE, Crowley WF, Jr. & Padmanabhan V. (1998). A two-site chemiluminescent assay for activin-free follistatin reveals that most follistatin circulating in men and normal cycling women is in an activin-bound state. *The Journal of Clinical Endocrinology and Metabolism* **83,** 851-858.

McCracken JA, Glew ME & Scaramuzzi RJ. (1970). Corpus luteum regression induced by prostagland in F2-alpha. *The Journal of Clinical Endocrinology and Metabolism* **30,** 544-546.

McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H & Hsueh AJW. (1997). Preantral ovarian follicles in serum-free culture: Suppression of apoptosis after activation of the cyclic guanosine 3',5'- monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology* **138,** 2417-2424.

McGee EA & Hsueh AJW. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews* **21,** 200-214.

McLeod BJ, Haresign W & Lamming GE. (1982). The Induction of Ovulation and Luteal Function in Seasonally Anestrous Ewes Treated with Small-Dose Multiple Injections of GnRH. *Journal of Reproduction and Fertility* **65,** 215-221.

McNatty KP. (1978). Follicular fluid. In *The Vertebrate Ovary*, ed. Jones R, pp. 215-259. Plenum, New York.

McNatty KP, Hudson N, Gibb M, Ball K, Henderson KM, Heath DA, Lun S & Kieboom LE. (1985). FSH influences follicle viability, oestradiol biosynthesis and ovulation rate in Romney ewes. *Journal of Reproduction and Fertility* **75,** 121-131.

McNatty KP & Henderson KM. (1987). Gonadotrophins, fecundity genes and ovarian follicular function. *Journal of Steroid Biochemistry* **27,** 365-373.

McNatty KP, Heath DA, Hudson N & Clarke IJ. (1990). Effect of Long-Term Hypophysectomy on Ovarian Follicle Populations and Gonadotropin-Induced Adenosine Cyclic 3',5'- Monophosphate Output by Follicles from Booroola Ewes with or without the F Gene. *Journal of Reproduction and Fertility* **90,** 515-522.

McNatty KP, Smith P, Hudson NL, Heath DA, Lun S & O WS. (1992). Follicular development and steroidogenesis. In *Local Regulation of Ovarian Function*, ed. Sjoberg N, Hamberger L, Jansen PO, Owman C & Coelingh HIJ, pp. 21-28. Parthenon Publishing Group, Carnforth.

McNatty KP, Hudson NL, Heath DA, Shaw L, Blay L, Berry L & Lun S. (1993). Effect of chronic FSH administration on ovarian follicular development, ovulation rate and corpora lutea formation in sheep. *The Journal of Endocrinology* **138,** 315-325.

McNeilly AS. (1984). Changes in FSH and the pulsatile secretion of LH during the delay in oestrus induced by treatment of ewes with bovine follicular fluid. *Journal of Reproduction and Fertility* **72,** 165-172.

McNeilly AS, Crow W, Brooks J & Evans G. (1992). Luteinizing Hormone Pulses, Follicle-Stimulating Hormone and Control of Follicle Selection in Sheep. *Journal of Reproduction and Fertility* **Suppl. 45,** 5-19.

Meidan R, Aberdam E & Aflalo L. (1992). Steroidogenic enzyme content and progesterone induction by cyclic adenosine 3',5'-monophosphate-generating agents and prostaglandin F2 alpha in bovine theca and granulosa cells luteinized in vitro. *Biology of Reproduction* **46,** 786-792.

Mihm M, Curran N, Hyttel P, Knight PG, Boland MP & Roche JF. (1999). Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. *Journal of Reproduction and Fertility* **116,** 293-304.

Mihm M & Austin EJ. (2002). The final stages of dominant follicle selection in cattle. *Domestic Animal Endocrinology* **23,** 155-166.

Mihm M, Baker PJ, Ireland JL, Smith GW, Coussens PM, Evans AC & Ireland JJ. (2006). Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle. *Biology of Reproduction* **74,** 1051-1059.

Mihm M, Baker PJ, Fleming LM, Monteiro AM & O'Shaughnessy PJ. (2008). Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. *Reproduction (Cambridge, England)* **135,** 253-265.

Miller KF, Critser JK, Rowe RF & Ginther OJ. (1979a). Ovarian effects of bovine follicular fluid treatment in sheep and cattle. *Biology of Reproduction* **21,** 537-544.

Miller WL. (2007). StAR search--what we know about how the steroidogenic acute regulatory protein mediates mitochondrial cholesterol import. *Molecular Endocrinology (Baltimore), Md* **21,** 589-601.

Miro F & Hillier SG. (1992). Relative effects of activin and inhibin on steroid hormone synthesis in primate granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **75,** 1556-1561.

Miro F & Hillier SG. (1996). Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. *Endocrinology* **137,** 464-468.

Moenter SM, Caraty A, Locatelli A & Karsch FJ. (1991). Pattern of gonadotropin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge. *Endocrinology* **129,** 1175-1182.

Moenter SM, Caraty A, Lehman MN & Karsch FJ. (1993). Characterization and regulation of pre-ovulatory secretion of gonadotrophin-releasing hormone. *Human Reproduction (Oxford, England)* **8 Suppl 2,** 51-56.

Monniaux D, Mandon-Pepin B & Monget P. (1999). Follicular atresia, a programmed wastage. *M S - Medecine Sciences* **15,** 157-166.

Moulton BC. (1994). Transforming growth factor-beta stimulates endometrial stromal apoptosis in vitro. *Endocrinology* **134,** 1055-1060.

Murdoch WJ & Dunn TG. (1982). Alterations in follicular steroid hormones during the preovulatory period in the ewe. *Biology of Reproduction* **27,** 300-307.

Nakatani A, Shimasaki S, Depaolo LV, Erickson GF & Ling N. (1991). Cyclic Changes in Follistatin Messenger Ribonucleic Acid and Its Protein in the Rat Ovary During the Estrous Cycle. *Endocrinology* **129,** 603-611.

Nelson VL, Legro RS, Strauss JF, 3rd & McAllister JM. (1999). Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. *Molecular Endocrinology (Baltimore), Md* **13,** 946-957.

Nelson VL, Qin KN, Rosenfield RL, Wood JR, Penning TM, Legro RS, Strauss JF, 3rd & McAllister JM. (2001). The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism* **86,** 5925-5933.

Nimz M, Spitschak M, Schneider F, Furbass R & Vanselow J. (2009). Down-regulation of genes encoding steroidogenic enzymes and hormone receptors in late preovulatory follicles of the cow coincides with an accumulation of intrafollicular steroids. *Domestic Animal Endocrinology* **37,** 45-54.

Nishimori K & Matzuk MM. (1996). Transgenic mice in the analysis of reproductive development and function. *Reviews of Reproduction* **1,** 203-212.

Norman RJ, Dewailly D, Legro RS & Hickey TE. (2007). Polycystic ovary syndrome. *Lancet* **370,** 685-697.

O'Shaughnessy PJ, McLelland D & McBride MW. (1997). Regulation of luteinizing hormone receptor and follicle-stimulating hormone receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. *Biology of Reproduction* **57,** 602-608.

Oktay K, Briggs D & Gosden RG. (1997). Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *Journal of Clinical Endocrinology and Metabolism* **82,** 3748-3751.

Ong KK & Dunger DB. (2002). Perinatal growth failure: the road to obesity, insulin resistance and cardiovascular disease in adults. *Best Practice & Research* **16,** 191-207.

Ortega HH, Salvetti NR & Padmanabhan V. (2009). Developmental programming: prenatal androgen excess disrupts ovarian steroid receptor balance. *Reproduction (Cambridge, England).*

Osman P. (1985). Rate and course of atresia during follicular development in the adult cyclic rat. *Journal of Reproduction and Fertility* **73,** 261-270.

Otsuka F, Yao ZX, Lee TH, Yamamoto S, Erickson GF & Shimasaki S. (2000). Bone morphogenetic protein-15. Identification of target cells and biological functions. *Journal of Biological Chemistry* **275,** 39523-39528.

Packer AI, Hsu YC, Besmer P & Bacharova RF. (1994). The ligand of the c-kit receptor promotes oocyte growth. *Developmental Biology* **161,** 194-205.

Padmanabhan V, Evans NP, Dahl GE, McFadden KL, Mauger DT & Karsch FJ. (1995). Evidence for short or ultrashort loop negative feedback of gonadotropin-releasing hormone secretion. *Neuroendocrinology* **62,** 248-258.

Padmanabhan V, Battaglia D, Brown MB, Karsch FJ, Lee JS, Pan W, Phillips DJ & Van Cleeff J. (2002). Neuroendocrine control of follicle-stimulating hormone (FSH) secretion: II. Is follistatin-induced suppression of FSH secretion mediated via changes in activin availability and does it involve changes in gonadotropin-releasing hormone secretion? *Biology of Reproduction* **66,** 1395-1402.

Padmanabhan V, Manikkam M, Recabarren S & Foster D. (2006). Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. *Molecular and cellular endocrinology* **246,** 165-174.

Pant HC, Hopkinson CR & Fitzpatrick RJ. (1977). Concentration of oestradiol, progesterone, luteinizing hormone and follicle-stimulating hormone in the jugular venous plasma of ewes during the oestrous cycle. *The Journal of Endocrinology* **73,** 247-255.

Paton AC & Collins WP. (1992). Differentiation processes of granulosa cells. In *Oxford Reviews of Reproductive Biology*, ed. Milligan SR, pp. 169-223. Oxford University Press, Oxford.

Pau KY, Berria M, Hess DL & Spies HG. (1993). Preovulatory gonadotropin-releasing hormone surge in ovarian-intact rhesus macaques. *Endocrinology* **133,** 1650-1656.

Pelletier G, Labrie C & Labrie F. (2000). Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. *The Journal of Endocrinology* **165,** 359-370.

Peluso JJ & Pappalardo A. (1998). Progesterone mediates its anti-mitogenic and anti-apoptotic actions in rat granulosa cells through a progesterone-binding protein with gamma aminobutyric acidA receptor-like features. *Biology of Reproduction* **58,** 1131-1137.

Peluso JJ. (2006). Multiplicity of progesterone's actions and receptors in the mammalian ovary. *Biology of Reproduction* **75,** 2-8.

Peters H. (1979). Some aspects of early follicular development. In *Ovarian follicular development and function*, ed. Midgley AR & Sadler WA, pp. 1-13. Raven Press, New York.

Phillips DJ, Hudson NL, Gentle LR & McNatty KP. (1994). Bioactive follicle-stimulating hormone concentrations in plasma during the estrous cycle of the ewe. *Biology of Reproduction* **51,** 1292-1298.

Picton HM, Tsonis CG & McNeilly AS. (1990). The Antagonistic Effect of Exogenous LH Pulses on FSH-Stimulated Preovulatory Follicle Growth in Ewes Chronically Treated with a Gonadotropin-Releasing Hormone Agonist. *Journal of Endocrinology* **127,** 273-283.

Portela VM, Zamberlam G & Price CA. (2010). Cell plating density alters the ratio of estrogenic to progestagenic enzyme gene expression in cultured granulosa cells. *Fertility and Sterility* **93,** 2050-2055.

Qi Y, Oldfield BJ & Clarke IJ. (2009). Projections of RFamide-related peptide-3 neurones in the ovine hypothalamus, with special reference to regions regulating energy balance and Reproduction. *Journal of Neuroendocrinology* **21,** 690-697.

Rae MT, Palassio S, Kyle CE, Brooks AN, Lea RG, Miller DW & Rhind SM. (2001). Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction* **122,** 915-922.

Rai NK, Tripathi K, Sharma D & Shukla VK. (2005). Apoptosis: a basic physiologic process in wound healing. *The International Journal of Lower Extremity Wounds* **4,** 138-144.

Rannikki AS, Zhang FP & Huhtaniemi IT. (1995). Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. *Molecular and Cellular Endocrinology* **107,** 199-208.

Rath-Coursey K, Bove S, Nishibori M & Pate J. (1999). Localization of macrophage migration inhibitory factor (MIF) and expression of MIF mRNA in the ovary and uterus of pregnant and nonpregnant sheep. *Biology of Reproduction* **60,** 219.

Ratts VS, Flaws JA, Kolp R, Sorenson CM & Tilly JL. (1995). Ablation of *bcl-2* gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology* **136,** 3665-3668.

Rawlings NC & Cook SJ. (1993). LH-secretion around the time of the preovulatory gonadotropin surge in the ewe. *Animal Reproduction Science* **30,** 289-299.

Rawlings NC, Evans AC, Honaramooz A & Bartlewski PM. (2003). Antral follicle growth and endocrine changes in prepubertal cattle, sheep and goats. *Animal Reproduction Science* **78,** 259-270.

Rebar R, Judd HL, Yen SS, Rakoff J, Vandenberg G & Naftolin F. (1976). Characterization of the inappropriate gonadotropin secretion in polycystic ovary syndrome. *The Journal of Clinical Investigation* **57,** 1320-1329.

Recabarren SE, Padmanabhan V, Codner E, Lobos A, Duran C, Vidal M, Foster DL & Sir-Petermann T. (2005). Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. *American Journal of Physiology* **289,** E801-806.

Reilly CM, Cannady WE, Mahesh VB, Stopper VS, DeSevilla LM & Mills TM. (1996). Duration of estrogen exposure prior to follicle-stimulating hormone stimulation is critical to granulosa cell growth and differentiation in rats. *Biology of Reproduction* **54,** 1336-1342.

Reynaud K, Cortvrindt R, Smitz J & Driancourt MA. (2000). Effects of Kit Ligand and anti-Kit antibody on growth of cultured mouse preantral follicles. *Molecular Reproduction and Development* **56,** 483-494.

Rhind SM, Rae MT & Brooks AN. (2001). Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction (Cambridge, England)* **122,** 205-214.

Richards JS, Jahnsen T, Hedin L, Lifka L, Ratoosh S, Durica JM & Goldring NB. (1987). Ovarian follicular development: from physiology to molecular biology. *Recent Progress in Hormone Research* **43,** 231-276.

Richards JS & Hedin L. (1988). Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. *Annual Review of Physiology* **50,** 441-463.

Richards JS. (1994). Hormonal control of gene expression in the ovary. *Endocrine Reviews* **15,** 725-751.

Richards JS, Russell DL, Robker RL, Dajee M & Alliston TN. (1998). Molecular mechanisms of ovulation and luteinization. *Molecular and Cellular Endocrinology* **145,** 47-54.

Roberson MS, Wolfe MW, Stumpf TT, Kittok RJ & Kinder JE. (1989). Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. *Biology of Reproduction* **41,** 997-1003.

Roberts AJ & Skinner MK. (1990). Estrogen regulation of thecal cell steroidogenesis and differentiation: thecal cell-granulosa cell interactions. *Endocrinology* **127,** 2918-2929.

Roberts VJ, Barth S, el-Roeiy A & Yen SS. (1993). Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. *The Journal of Clinical Endocrinology and Metabolism* **77,** 1402-1410.

Robinson JE & Karsch FJ. (1984). Refractoriness to inductive day lengths terminates the breeding season of the Suffolk ewe. *Biology of Reproduction* **31,** 656-663.

Robinson JE, Forsdike RA & Taylor JA. (1999). In utero exposure of female lambs to testosterone reduces the sensitivity of the gonadotropin-releasing hormone neuronal network to inhibition by progesterone. *Endocrinology* **140,** 5797-5805.

Robinson JJ. (1996). Nutrition and Reproduction. *Animal Reproduction Science* **42,** 25-34.

Robinson R. (2001). The fetal origins of adult disease. *BMJ (Clinical research ed)* **322,** 375-376.

Robker RL & Richards JS. (1998). Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Molecular endocrinology (Baltimore), Md* **12,** 924-940.

Rodgers RJ & Irving-Rodgers HF. (2009). Morphological classification of bovine ovarian follicles. *Reproduction (Cambridge, England)* **139,** 309-318.

Rolaki A, Drakakis P, Millingos S, Loutradis D & Makrigiannakis A. (2005). Novel trends in follicular development, atresia and corpus luteum regression: a role for apoptosis. *Reproductive Biomedicine Online* **11,** 93-103.

Rosenfeld CS, Wagner JS, Roberts RM & Lubahn DB. (2001). Intraovarian actions of oestrogen. *Reproduction (Cambridge, England)* **122,** 215-226.

Rosser C, Herkimer C, Sarma HN, Recabarren SE, Dunaif A & Padmanabhan V. (2003). Fetal programming: prenatal exposure to excess testosterone programs hyperinsulinemia. *Biology of Reproduction 68(Suppl 1):293 (Abstract 440)*

Rueda BR, Hendry IR, Tilly JL & Hamernik DL. (1999). Accumulation of caspase-3 messenger ribonucleic acid and induction of caspase activity in the ovine corpus luteum following prostaglandin F2alpha treatment in vivo. *Biology of Reproduction* **60,** 1087-1092.

Ryan KE, Casey SM, Canty MJ, Crowe MA, Martin F & Evans AC. (2007). Akt and Erk signal transduction pathways are early markers of differentiation in dominant and subordinate ovarian follicles in cattle. *Reproduction (Cambridge, England)* **133,** 617-626.

Ryan KE, Glister C, Lonergan P, Martin F, Knight PG & Evans AC. (2008). Functional significance of the signal transduction pathways Akt and Erk in ovarian follicles: in vitro and in vivo studies in cattle and sheep. *Journal of Ovarian Research* **1,** 2.

Sarkar DK, Chiappa SA, Fink G & Sherwood NM. (1976). Gonadotropin-releasing hormone surge in pro-oestrous rats. *Nature* **264,** 461-463.

Sarma HN, Manikkam M, Herkimer C, Dell'Orco J, Welch KB, Foster DL & Padmanabhan V. (2005). Fetal programming: excess prenatal testosterone reduces postnatal luteinizing hormone, but not follicle-stimulating hormone responsiveness, to estradiol negative feedback in the female. *Endocrinology* **146,** 4281-4291.

Savabieasfahani M, Lee JS, Herkimer C, Sharma TP, Foster DL & Padmanabhan V. (2005). Fetal programming: testosterone exposure of the female sheep during midgestation disrupts the dynamics of its adult gonadotropin secretion during the periovulatory period. *Biology of Reproduction* **72,** 221-229.

Sawyer HR, Smith P, Heath DA, Juengel JL, Wakefield SJ & McNatty KP. (2002). Formation of ovarian follicles during fetal development in sheep. *Biology of Reproduction* **66,** 1134-1150.

Scaramuzzi RJ, Turnball KE & Nancarrow CD. (1980). Growth of Graafian follicles in cows following luteolysis induced by the prostaglandin F2α analogue, cloprostenol. *Australian Journal of Biological Science* **33,** 63-69.

Scaramuzzi RJ, Adams NR, Baird DT, Campbell BK, Downing JA, Findlay JK, Henderson KM, Martin GB, McNatty KP, McNeilly AS & et al. (1993). A model for follicle selection and the determination of ovulation rate in the ewe. *Reproduction, Fertility, and Development* **5,** 459-478.

Schams D & Berisha B. (2002). Steroids as local regulators of ovarian activity in domestic animals. *Domestic Animal Endocrinology* **23,** 53-65.

Schmittgen TD & Livak KJ. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* **3,** 1101-1108.

Schreiber JR, Nakamura K & Erickson GF. (1980). Progestins inhibit FSH-stimulated steroidogenesis in cultured rat granulosa cells. *Molecular and Cellular Endocrinology* **19,** 165-173.

Schwanzel-Fukuda M & Pfaff DW. (1989). Origin of luteinizing hormone-releasing hormone neurons. *Nature* **338,** 161-164.

Shao R, Markstrom E, Friberg PA, Johansson M & Billig H. (2003). Expression of progesterone receptor (PR) A and B isoforms in mouse granulosa cells: stage-dependent PR-mediated regulation of apoptosis and cell proliferation. *Biology of Reproduction* **68,** 914-921.

Sharma TP, Herkimer C, West C, Ye W, Birch R, Robinson JE, Foster DL & Padmanabhan V. (2002). Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. *Biology of Reproduction* **66,** 924-933.

Sheffield JW & O'Shaughnessy PJ. (1989). Effect of injection of gonadotrophin-releasing hormone on testicular steroidogenesis in the hypogonadal (hpg) mouse. *Journal of Reproduction and Fertility* **86,** 609-617.

Shimasaki S, Koga M, Esch F, Cooksey K, Mercado M, Koba A, Ueno N, Ying SY, Ling N & Guillemin R. (1988). Primary structure of the human follistatin precursor and its genomic organization. *Proceedings of the National Academy of Sciences of the United States of America* **85,** 4218-4222.

Shimasaki S, Koga M, Buscaglia ML, Simmons DM, Bicsak TA & Ling N. (1989). Follistatin Gene Expression in the Ovary and Extragonadal Tissues. *Molecular Endocrinology* **3,** 651-659.

Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ & Weinberg RA. (1996). Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* **384,** 470-474.

Sidis Y, Fujiwara T, Leykin L, Isaacson K, Toth T & Schneyer AL. (1998). Characterization of inhibin/activin subunit, activin receptor, and follistatin messenger ribonucleic acid in human and mouse oocytes: Evidence for activin's paracrine signaling from granulosa cells to oocytes. *Biology of Reproduction* **59,** 807-812.

Silva JM, Hamel M, Sahmi M & Price CA. (2006). Control of oestradiol secretion and of cytochrome P450 aromatase messenger ribonucleic acid accumulation by FSH involves different intracellular pathways in oestrogenic bovine granulosa cells in vitro. *Reproduction (Cambridge, England)* **132,** 909-917.

Silverman A-J, Livne I & JW W. (1994). The gonadotropin-releasing hormone (GnRH) neuronal systems: Immunochemistry and in situ hybridization. *In: Knobil E, Neill JD, editors Physiology of Reproduction New York: Raven Press; 1994 p 1683-710.*

Simoni M, Gromoll J & Nieschlag E. (1997). The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocrine Reviews* **18,** 739-773.

Sisk CL, Richardson HN, Chappell PE & Levine JE. (2001). In vivo gonadotropin-releasing hormone secretion in female rats during peripubertal development and on proestrus. *Endocrinology* **142,** 2929-2936.

Skinner DC, Malpaux B, Delaleu B & Caraty A. (1995). Luteinizing hormone (LH)-releasing hormone in third ventricular cerebrospinal fluid of the ewe: correlation with LH pulses and the LH surge. *Endocrinology* **136,** 3230-3237.

Sklar CA, Mueller PL, Gluckman PD, Kaplan SL, Rudolph AM & Grumbach MM. (1981). Hormone ontogeny in the ovine fetus. VII. Circulating luteinizing hormone and follicle-stimulating hormone in mid- and late gestation. *Endocrinology* **108,** 874-880.

Smith MF, Gutierrez CG, Ricke WA, Armstrong DG & Webb R. (2005). Production of matrix metalloproteinases by cultured bovine theca and granulosa cells. *Reproduction (Cambridge, England)* **129,** 75-87.

Smith P, O WS, Hudson NL, Shaw L, Heath DA, Condell L, Phillips DJ & McNatty KP. (1993). Effects of the Booroola Gene (Fec^B) on Body Weight, Ovarian Development and Hormone Concentrations During Fetal Life. *Journal of Reproduction and Fertility* **98,** 41-54.

Smith P, O WS, Corrigan KA, Smith T, Lundy T, Davis GH & McNatty KP. (1997). Ovarian morphology and endocrine characteristics of female sheep fetuses that are heterozygous or homozygous for the Inverdale prolificacy gene (*fecXI*). *Biology of Reproduction* **57,** 1183-1192.

Smith P, Steckler TL, Veiga-Lopez A & Padmanabhan V. (2008). Developmental Programming: Differential Effects of Prenatal Testosterone and Dihydrotestosterone on Follicular Recruitment, Depletion of Follicular Reserve, and Ovarian Morphology in Sheep. *Biology of Reproduction.*

Souza CJ, Campbell BK & Baird DT. (1997). Follicular dynamics and ovarian steroid secretion in sheep during the follicular and early luteal phases of the estrous cycle. *Biology of Reproduction* **56,** 483-488.

Souza CJ, Campbell BK & Baird DT. (1998). Follicular waves and concentrations of steroids and inhibin A in ovarian venous blood during the luteal phase of the oestrous cycle in ewes with an ovarian autotransplant. *The Journal of Endocrinology* **156,** 563-572.

Spicer LJ, Alpizar E & Echternkamp SE. (1993). Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and(or) insulin-like growth factor I production in vitro. *Journal of Animal Science* **71,** 1232-1241.

Steckler T, Wang J, Bartol FF, Roy SK & Padmanabhan V. (2005). Fetal programming: prenatal testosterone treatment causes intrauterine growth retardation, reduces ovarian reserve and increases ovarian follicular recruitment. *Endocrinology* **146,** 3185-3193.

Steckler T, Manikkam M, Inskeep EK & Padmanabhan V. (2007a). Developmental programming: follicular persistence in prenatal testosterone-treated sheep is not programmed by androgenic actions of testosterone. *Endocrinology* **148,** 3532-3540.

Steckler TL, Roberts EK, Doop DD, Lee TM & Padmanabhan V. (2007b). Developmental programming in sheep: administration of testosterone during 60-90 days of pregnancy reduces breeding success and pregnancy outcome. *Theriogenology* **67,** 459-467.

Steckler TL, Lee JS, Ye W, Inskeep EK & Padmanabhan V. (2008). Developmental programming: exogenous gonadotropin treatment rescues ovulatory function but does not completely normalize ovarian function in sheep treated prenatally with testosterone. *Biology of Reproduction* **79,** 686-695.

Steckler TL, Herkimer C, Dumesic DA & Padmanabhan V. (2009). Developmental programming: excess weight gain amplifies the effects of prenatal testosterone excess on reproductive cyclicity--implication for polycystic ovary syndrome. *Endocrinology* **150,** 1456-1465.

Steiner RA, Clifton DK, Spies HG & Resko JA. (1976). Sexual differentiation and feedback control of luteinizing hormone secretion in the rhesus monkey. *Biology of Reproduction* **15,** 206-212.

Stocco DM & Clark BJ. (1996). Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17,** 221-244.

Stocco DM. (2000). Intramitochondrial cholesterol transfer. *Biochimica et Biophysica Acta* **1486,** 184-197.

Strauss JF, 3rd, Kallen CB, Christenson LK, Watari H, Devoto L, Arakane F, Kiriakidou M & Sugawara T. (1999). The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. *Recent Progress in Hormone Research* **54,** 369-394; discussion 394-365.

Strauss JF, Schuler LA, Rosenblum MF & Tanaka T. (1981). Cholesterol metabolism by ovarian tissue. *Advances in Lipid Research* **18,** 99-157.

Strauss JF & Penning TM. (1999). Synthesis of the sex steroid hormones: molecular and structural biology with applications to Clinical practice. In *Molecular Biology in Reproductive Medicine*, ed. Fauser BCJM, Rutherford AJ, Strauss JF & Van Steirteghem A, pp. 201-232. Parthenon Publishing, Carnforth.

Sugino K, Kurosawa N, Nakamura T, Takio K, Shimasaki S, Ling N, Titani K & Sugino H. (1993). Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. *The Journal of Biological Chemistry* **268,** 15579-15587.

Sullivan SD & Moenter SM. (2004). Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common Fertility disorder. *Proceedings of the National Academy of Sciences of the United States of America* **101,** 7129-7134.

Sunderland SJ, Crowe MA, Boland MP, Roche JF & Ireland JJ. (1994). Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *Journal of Reproduction and Fertility* **101,** 547-555.

Svensson EC, Markstrom E, Andersson M & Billig H. (2000). Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human chorionic gonadotropin. *Biology of Reproduction* **63,** 1457-1464.

Taylor AH & Al-Azzawi F. (2000). Immunolocalisation of oestrogen receptor beta in human tissues. *Journal of Molecular Endocrinology* **24,** 145-155.

Tchernitchin AN, Tchernitchin NN, Mena MA, Unda C & Soto J. (1999). Imprinting: perinatal exposures cause the development of diseases during the adult age. *Acta Biologica Hungarica* **50,** 425-440.

Terqui M, Berthelot F & Driancourt MA. (1988). Morphological and Functional Features of Ovine Follicles in Perifusion with Pulsatile Hormone Delivery. *Reproduction Nutrition Development* **28,** 1093-1104.

Tetsuka M & Nancarrow CD. (2007). The levels of 5alpha-dihydrotestosterone in follicular fluid in healthy and atretic ovine follicles. *Domestic Animal Endocrinology* **33,** 347-357.

Thomas GB, McNeilly AS & Brooks AN. (1993). Development of gonadotrophs and thyrotrophs in the female foetal sheep pituitary: immunocytochemical localization studies. *Journal of Neuroendocrinology* **5,** 157-161.

Thornberry NA & Lazebnik Y. (1998). Caspases: enemies within. *Science (New York, NY* **281,** 1312-1316.

Tilly JL, Kowalski KI, Johnson AL & Hsueh AJ. (1991). Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* **129,** 2799-2801.

Tilly JL, Tilly KI, Kenton ML & Johnson AL. (1995). Expression of members of the *bcl-2* gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of apoptosis is associated with decreased *bax* and constitutive *bcl-2* and *bcl-x*$_{long}$ messenger ribonucleic acid levels. *Endocrinology* **136,** 232-241.

Tisdall DJ, Watanabe K, Hudson NL, Smith P & McNatty KP. (1995). FSH Receptor Gene Expression During Ovarian Follicle Development in Sheep. *Journal of Molecular Endocrinology* **15,** 273-281.

Tsai NM, Hsieh RH, Au HK, Shieh MJ, Huang SY & Tzeng CR. (2005). Effects of gonadotrophin-releasing hormone agonists on apoptosis of granulosa cells. *Annals of the New York Academy of Sciences* **1042,** 531-537.

Tsilchorozidou T, Overton C & Conway GS. (2004). The pathophysiology of polycystic ovary syndrome. *Clinical Endocrinology* **60,** 1-17.

Tsonis CG, Carson RS & Findlay JK. (1984). Relationships between aromatase activity, follicular fluid oestradiol-17 beta and testosterone concentrations, and diameter and atresia of individual ovine follicles. *Journal of Reproduction and Fertility* **72,** 153-163.

Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, Ishii S & Sharp PJ. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and Biophysical Research Communications* **275,** 661-667.

Turzillo AM & Fortune JE. (1990). Suppression of the Secondary FSH Surge with Bovine Follicular Fluid Is Associated with Delayed Ovarian Follicular Development in Heifers. *Journal of Reproduction and Fertility* **89,** 643-653.

Unsworth WP, Taylor JA & Robinson JE. (2005). Prenatal programming of reproductive neuroendocrine function: the effect of prenatal androgens on the development of estrogen positive feedback and ovarian cycles in the ewe. *Biology of Reproduction* **72,** 619-627.

Van den Hurk R, Dijkstra G, Van Mil FN, Hulshof SCJ & Van den Ingh TSGAM. (1995). Distribution of the intermediate filament proteins vimentin, keratin, and desmin in the bovine ovary. *Molecular Reproduction and Development* **41,** 459-467.

Van den Hurk R, Bevers MM & Beckers JF. (1997). In-vivo and in-vitro development of preantral follicles. *Theriogenology* **47,** 73-82.

VanGuilder HD, Vrana KE & Freeman WM. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *BioTechniques* **44,** 619-626.

Vanholder T, Opsomer G & de Kruif A. (2006). Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. *Reproduction, Nutrition, Development* **46,** 105-119.

Veiga-Lopez A, Ye W, Phillips DJ, Herkimer C, Knight PG & Padmanabhan V. (2008). Developmental programming: deficits in reproductive hormone dynamics and ovulatory outcomes in prenatal, testosterone-treated sheep. *Biology of Reproduction* **78,** 636-647.

Veiga-Lopez A, Astapova OI, Aizenberg EF, Lee JS & Padmanabhan V. (2009). Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biology of Reproduction* **80,** 718-725.

Vitt UA, Hayashi M, Klein C & Hsueh AJW. (2000a). Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biology of Reproduction* **62,** 370-377.

Vitt UA, McGee EA, Hayashi M & Hsueh AJW. (2000b). In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology* **141,** 3814-3820.

Walker WA & Humphries C. (2007). Starting the good life in the womb. *Newsweek* **150,** 56, 58.

Wandji SA, Srsen V, Voss AK, Eppig JJ & Fortune JE. (1996). Initiation *in vitro* of growth of bovine primordial follicles. *Biology of Reproduction* **55,** 942-948.

Wang XN & Greenwald GS. (1993). Hypophysectomy of the Cyclic Mouse .1. Effects on Folliculogenesis, Oocyte Growth, and Follicle-Stimulating Hormone and Human Chorionic Gonadotropin Receptors. *Biology of Reproduction* **48,** 585-594.

Webb R, Gong JG & Bramley TA. (1994). Role of Growth Hormone and Intrafollicular Peptides in Follicle Development in Cattle. *Theriogenology* **41,** 25-30.

Webb R, Campbell BK, Garverick HA, Gong JG, Gutierrez CG & Armstrong DG. (1999a). Molecular mechanisms regulating follicular recruitment and selection. *Journal of Reproduction and Fertility Supplement* **54,** 33-48.

Webb R, Gosden RG, Telfer EE & Moor RM. (1999b). Factors affecting folliculogenesis in ruminants. *Animal Science* **68,** 257-284.

Webb R, Nicholas B, Gong JG, Campbell BK, Gutierrez CG, Garverick HA & Armstrong DG. (2003). Mechanisms regulating follicular development and selection of the dominant follicle. *Reproduction (Cambridge, England) Supplement* **61,** 71-90.

Webb R & Campbell BK. (2007). Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Society of Reproduction and Fertility Supplement* **64,** 141-163.

Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K & Franks S. (2003). Formation and early development of follicles in the polycystic ovary. *Lancet* **362,** 1017-1021.

Weiser MJ, Foradori CD & Handa RJ. (2008). Estrogen receptor beta in the brain: from form to function. *Brain research reviews* **57,** 309-320.

West C, Foster DL, Evans NP, Robinson J & Padmanabhan V. (2001). Intra-follicular activin availability is altered in prenatally-androgenized lambs. *Molecular and Cellular Endocrinology* **185,** 51-59.

Wickenheisser JK, Quinn PG, Nelson VL, Legro RS, Strauss JF, 3rd & McAllister JM. (2000). Differential activity of the cytochrome P450 17alpha-hydroxylase and steroidogenic acute regulatory protein gene promoters in normal and polycystic ovary syndrome theca cells. *The Journal of Clinical Endocrinology and Metabolism* **85,** 2304-2311.

Wildt L, Hausler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE & Knobil E. (1981). Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. *Endocrinology* **109,** 376-385.

Williams GT, Smith CA, Spooncer E & Dexter TM. (1990). Haematopoietic colony stimulating factors promote cell survival by supressing apoptosis. *Nature* **343,** 76-79.

Withers PC. (1992). Comparative animal physiology. *Harcourt Brace Jovanovich College, Orlando.*

Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA & Gray LE, Jr. (2002). Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. *Toxicology Science* **65,** 71-86.

Wood RI, Mehta V, Herbosa CG & Foster DL. (1995). Prenatal testosterone differentially masculinizes tonic and surge modes of luteinizing hormone secretion in the developing sheep. *Neuroendocrinology* **62,** 238-247.

Wood RI & Foster DL. (1998). Sexual differentiation of reproductive neuroendocrine function in sheep. *Reviews of Reproduction* **3,** 130-140.

Wood RI, Robinson JE, Forsdike RA, Padmanabhan V & Foster DL. (2000). Sexual differentiation of the timing of sexual maturation in sheep. In J.-P. Bourguignon & T. M. Plant. sheep. In J.-P. Bourguignon & T. M. Plant (Eds.). *The onset of puberty in perspective (pp 269–287).*

Wrathall JHM & Knight PG. (1995). Effects of inhibin-related peptides and oestradiol on androstenedione and progesterone secretion by bovine theca cells in vitro. *Journal of Endocrinology* **145,** 491-500.

Xiao S, Farnworth PG & Findlay JK. (1992a). Interaction between Activin and Follicle-Stimulating Hormone- Suppressing Protein Follistatin in the Regulation of Basal Inhibin Production by Cultured Rat Granulosa-Cells. *Endocrinology* **131,** 2365-2370.

Xiao S, Robertson DM & Findlay JK. (1992b). Effects of Activin and Follicle-Stimulating Hormone (FSH)-Suppressing Protein/Follistatin on FSH Receptors and Differentiation of Cultured Rat Granulosa Cells. *Endocrinology* **131,** 1009-1016.

Xita N & Tsatsoulis A. (2006). Review: fetal programming of polycystic ovary syndrome by androgen excess: evidence from experimental, Clinical, and genetic association studies. *The Journal of Clinical Endocrinology and Metabolism* **91,** 1660-1666.

Xu ZZ, Garverick HA, Smith GW, Smith MF, Hamilton SA & Youngquist RS. (1995a). Expression of Follicle-Stimulating Hormone and Luteinizing Hormone Receptor Messenger Ribonucleic Acids in Bovine Follicles During the First Follicular Wave. *Biology of Reproduction* **53,** 951-957.

Xu ZZ, Garverick HA, Smith GW, Smith MF, Hamilton SA & Youngquist RS. (1995b). Expression of Messenger Ribonucleic Acid Encoding Cytochrome P450 Side-Chain Cleavage, Cytochrome P450 17α-Hydroxylase, and Cytochrome P450 Aromatase in Bovine Follicles During the First Follicular Wave. *Endocrinology* **136,** 981-989.

Yacobi K, Wojtowicz A, Tsafriri A & Gross A. (2004). Gonadotropins enhance caspase-3 and -7 activity and apoptosis in the theca-interstitial cells of rat preovulatory follicles in culture. *Endocrinology* **145,** 1943-1951.

Yalow RS & Berson SA. (1960). Immunoassay of endogenous plasma insulin in man. *The Journal of Clinical Investigation* **39,** 1157-1175.

Yang MY & Rajamahendran R. (2000a). Involvement of apoptosis in the atresia of nonovulatory dominant follicle during the bovine estrous cycle. *Biology of Reproduction* **63,** 1313-1321.

Yang MY & Rajamahendran R. (2000b). Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured bovine granulosa cells. *Biology of Reproduction* **62,** 1209-1217.

Ying SY, Becker A, Ling N, Ueno N & Guillemin R. (1986). Inhibin and Beta Type Transforming Growth Factor (TGF-β) Have Opposite Modulating Effects on the Follicle-Stimulating Hormone (FSH)-Induced Aromatase Activity of Cultured Rat Granulosa Cells. *Biochemical and Biophysical Research Communications* **136,** 969-975.

Ying SY. (1988). Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Reviews* **9,** 267-293.

Yoshioka K, Suzuki C, Arai S, Iwamura S & Hirose H. (2001). Gonadotropin-releasing hormone in third ventricular cerebrospinal fluid of the heifer during the estrous cycle. *Biology of Reproduction* **64,** 563-570.

Youle RJ & Strasser A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews: Molecular and Cellular Biology* **9,** 47-59.

Yuan W, Lucy MC & Smith MF. (1996). mRNA for IGF-1, IGF-2, IGFBP-2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles. *Biology of Reproduction* **55,** 1045-1054.

Zeleznik AJ & Hillier SG. (1984). The role of gonadotropins in the selection of the preovulatory follicle. *Clinical Obstetrics and Gynecology* **27,** 927-940.

Zeleznik AJ & Kubik CJ. (1986). Ovarian responses in macaques to pulsatile infusion of follicle-stimulating hormone (FSH) and luteinizing hormone: increased sensitivity of the maturing follicle to FSH. *Endocrinology* **119,** 2025-2032.

Zhou R, Bird IM, Dumesic DA & Abbott DH. (2005). Adrenal hyperandrogenism is induced by fetal androgen excess in a rhesus monkey model of polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism* **90,** 6630-6637.